DISSECTING THE ROLE OF SPHINGOSINE 1-PHOSPHATE – SPHINGOSINE 1-PHOSPHATE RECEPTOR 1 IN INFLAMMATORY BONE REMODELLING

Lan Xiao

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Abstract

It is now well documented that the immune and skeletal systems are closely related and share many of the same regulatory pathways, such as cytokines, transcription factors and signalling pathways – a relationship that is now referred to as osteoimmunology. The immunomodulation of bone remodelling, a life-long and indispensable process in bone metabolism, is the foundation of this relationship. Bone remodelling is a process that consists of bone resorption, driven by osteoclasts, and bone formation, driven by osteoblasts, two states that are finely balanced under normal physiological conditions. The immune response under pathological conditions (i.e., inflammation) results in dysregulated bone remodelling in conditions such as rheumatoid arthritis (RA), periodontitis, and apical periodontitis. The study of immune modulation of bone remodelling under inflammatory conditions promises to give us a better understanding of the mechanisms responsible for the bone loss associated these diseases and opens the door to new therapeutic approaches.

Sphingosine-1-phosphate (S1P), together with its receptor, sphingosine-1-phosphate receptor 1 (S1PR1), acts as a regulator of both the immune response and bone remodelling processes and, therefore, plays a vital role in osteoimmunology. S1P-S1PR1 signalling induces both osteoclastogenesis and osteogenesis, which suggest it has an intriguing dual role in inflammatory bone remodelling. Whereas dysregulated S1P-S1PR1 signalling is linked to bone loss in destructive inflammatory bone diseases such as RA, its role during infections has not been studied in any detail. Consequently, very little is known about the role of S1P-S1PR1 signalling in infection-induced inflammatory bone remodelling, which forms the topic of this thesis.

In this thesis, the status of S1P-S1PR1 signalling in an infection-induced bone destructive disease, namely apical periodontitis, were investigated. It was noted that S1P-S1PR1 signalling was abnormally activated and associated with the expression of RANKL, a key factor in osteoclastogenesis. An animal model of apical periodontitis further confirmed the role of S1P-S1PR1 signalling in inducing infectious inflammatory bone loss.

Although there was a link between S1P-S1PR1 signalling and enhanced RANKL production to induced bone loss, it was still unknown what triggered the activation in

response to infection-induced inflammatory conditions. Using an *in vitro* co-culture system, the interaction between macrophages and bone marrow-derived mesenchymal stromal cells (BMSCs) in response to lipopolysaccharides (LPS) stimulation was found. Increased S1P production in BMSCs could activate S1PR1 and activated S1PR1 subsequently led to increased RANKL expression in BMSCs, which eventually resulted in induced osteoclastogenesis and bone loss.

Furthermore, since bone remodelling consists of osteoclastogenesis and osteogenesis, we sought to determine the status of osteogenesis under the effects of infection-induced inflammation environment. Interestingly, osteogenic markers were found to be upregulated in BMSCs co-cultured with macrophages, especially under the infection-induced inflammatory condition. Using siRNA techniques to block S1PR1 expression, it was demonstrated that osteogenesis was due to S1P-S1PR1 signalling activation in the inflammatory condition. Therefore, the interactions between macrophages and BMSCs could activate S1P-S1PR1 signalling and lead to increased osteogenesis in inflammatory conditions.

Taken together, the data from this study demonstrates that S1P-S1PR1 signalling is abnormally activated in infection-induced inflammation, which is likely to be partially due to the interactions between macrophages and BMSCs. The S1P-S1PR1 signalling appears to have a crucial role in inflammatory bone remodelling by stimulating RANKL expression in BMSCs and, therefore, facilitating osteoclastogenesis, while on the other hand also inducing osteogenesis. This study adds to our understanding of the pathogenesis of bone destruction or abnormal bone formation in infectious inflammatory diseases, knowledge that will aid in the future development of new therapies for the treatment of conditions such as periodontitis.

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List of Abbreviations

ALP	alkaline phosphatase
AP-1	activator protein1
Arg1	arginase-1
BMP-2	bone morphogenetic protein2
BMSCs	bone mesenchymal stem cells
BSP	bone sialoprotein
CERase	ceramidases
CM	conditioned medium
cDNA	complementary deoxyribonucleic acid
COL-1	type I collage
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CXCL12	chemokine (C-X-C motif) ligand 12
CXCR4	chemokine (C-X-C motif) receptor 4
DAB	3, 3'-diaminobenzidine
DCs	dendritic cells
DMEM	dulbecco's modified eagle medium
EDTA	ethylenediaminetetraacetic acid
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPNMB	glycoprotein non-metastatic melanoma protein B
GTPase	guanosine triphosphatase
Erk	extracellular signal regulated kinase
FBS	fetal bovine serum
FTY720	fingolimod

HGF	hepatocyte growth factor
HSCs	hematopoietic stem cells
HLA-G	human leukocyte antigen G
IDO	indoleamine 2,3-dioxygenase
IFNγ	interferon-γ
IGF	insulin-like growth factor
IHC	immunohistochemistry
IKK	inhibitor of nuclear factor kappa-B kinase
IL-1/4/6/10/12/13/17	interleukin-1, 4, 6, 10, 12, 13, 17
IL-1RA	IL-1 receptor antagonist
JNK	c-Jun N-terminal kinase
LIF	leukemia inhibitory factor
MAPK	mitogen-activated protein kinase
MCP1	monocyte chemotactic protein 1
M-CSF	macrophage colony-stimulating factor
MMP	matrixmetalloproteinase
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor kappa B
NFATc1	nuclear factor of activated T-cells, cytoplasmic 1
NKT cells	natural killer T cells
NO	nitric oxide
OA	osteoactivin
OCN	osteocalcin
OPG	osteoprotegerin
OPN	osteopontin
OSM	oncostatin M

OSMR	OSM receptor
PBS	phosphate buffered saline
PD-1	programmed death 1
PDGF	platelet-derived growth factor
PGE2	prostaglandin E2
PI3K	phosphoinositide 3-kinase
PLC	phospholipase C
P/S	penicillin and streptomycin
RA	rheumatoid arthritis
RANK	receptor activator of nuclear factor-kappa B
RANKL	receptor activator of nuclear factor factor-kappa B ligand
RNA	ribonucleic acid
ROS	reactive oxygen species
RUNX2	runt-related transcription factor 2
RT-qPCR	real time quantitative reverse transcription polymerase chain reaction
S1P	sphingosine-1-phosphate
S1PR1	sphingosine-1-phosphate receptor 1
SPL	S1P lyase
SPPs	S1P phosphatases
Smase	sphingomyelinases
STAT3	signal transducer and activator of transcription 3
TGF-β	transforming growth factor-β
Th cells	T helper cells
TLRs	toll-like receptors
TNF	tumour necrosis factor

TNAIP6	TNF-α-induced protein 6
TRAF6	tumour necrosis factor receptorassociated factor 6
TRAP	tartrate resistant acid phosphatase
Treg cells	regulatory T cells
µCT	micro-computed tomography

Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature: QUT Verified Signature

Date: March 2017

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Chapter 1: Introduction

1.1 BACKGROUND

The skeletal system undergoes a constant life-long process of adaptation, which consists of osteoclast-driven resorption and osteoblast-driven formation (Raggatt & Partridge, 2010). This dynamic turnover of bone is termed bone remodelling and plays a key role in maintaining bone strength and integrity (Hadjidakis & Androulakis, 2006). Under physiological conditions, bone remodelling is highly regulated and maintained in a balanced state by the immune system such that the amount of bone formation equals that of bone resorption (Arron & Choi, 2000). Under pathological conditions such as inflammation, this balance is disrupted; the over-activated immune response results in over-induced osteoclastogenesis and dysregulated osteogenesis, which eventually breaks the balance of remodelling, as seen in rheumatoid arthritis (RA) (Rodan & Martin, 2000), periodontitis (Taubman, Valverde, Han, & Kawai, 2005), and apical periodontitis (Wang & Stashenko, 1993). Therefore, studying this inflammatory response will help us reach a better understanding of the pathogenesis of destructive inflammatory bone diseases and the search for new therapeutic approaches for these diseases.

Among the immune-related factors, one of the most important sphingolipid metabolites, sphingosine-1-phosphate (S1P) together with its receptor sphingosine-1-phosphate receptor 1 (S1PR1), plays a vital role in the regulation of both the immune response and the bone remodelling process (Maceyka, Harikumar, Milstien, & Spiegel, 2012; Rivera, Proia, & Olivera, 2008). The S1P-S1PR1 signalling activates the immune response and hence facilitates osteoclastogenesis, however, it is also found to induce osteogenesis. This suggests that the role of S1P-S1PR1 in remodelling is more complicated and intriguing than first thought, especially under inflammatory conditions (Pederson, Ruan, Westendorf, Khosla, & Oursler, 2008; Sato, Iwasaki, Kitano, Tsunemi, & Sano, 2012). This signalling has been found to be abnormally activated in destructive inflammatory bone diseases such as RA which is involved in the aberrantly-induced immune response and osteoclastogenesis during the onset and development of RA (Kitano et al., 2006). The mechanism governing the role of S1P-S1PR1 in RA is still unclear and in need of further research, as is its the role of in

infection-induced bone loss. Invading pathogens have been found to affect the status of S1P-S1PR1 signalling and consequently lead to dysregulated immune modulation (Arish et al., 2015), further complicating the picture. Hence, it is of great importance to study the role of S1P-S1PR1 signalling in infection-induced inflammatory bone remodelling.

1.2 PURPOSES

The main purpose of this study is to investigate the involvement of S1P-S1PR1 signalling in infection-induced bone loss in apical periodontitis, to detect the cell-cell interaction (i.e. macrophages and bone marrow stem cells) based on S1P-S1PR1 signalling in infectious inflammatory condition, and to reveal the possible roles and mechanisms of this signalling in inflammatory bone remodelling. The main questions to be addressed are the follows:

- **What is the status of S1P-S1PR1 signalling in inflammatory destructive bone disease caused by infection? Is it related to the induced bone loss?**
- **What makes the status of this signalling abnormal under the infection-induced inflammatory condition?**
- **How does the S1P-S1PR1 signalling affect bone remodelling in the infection-induced inflammatory condition?**

1.3 POSSIBLE OUTCOMES AND SIGNIFICANCE

Despite many previous studies, it is still unclear how S1P-S1PR1signalling affects bone remodelling during inflammation, especially in the infection-induced diseases. Furthermore, it is not fully understood how this signalling becomes activated during inflammation and what kind of cells are involved in this activation. Based on this, it is hypothesized that the S1P-S1PR1 signalling is dysregulated during infection-induced bone destruction, which results in dysregulated bone remodelling under inflammatory conditions. This study will, therefore, shed some light on the pathogenesis of destructive bone diseases, and also provide potential new therapeutic approaches for the prevention and treatment of these diseases.

1.4 THESIS OUTLINE

The content of each chapter (except for *Chapter 1*) included in this thesis are listed below:

Chapter 2, is an in-depth review of the available literature on S1P-S1PR1 signalling and osteoimmunology (the interaction of the immune and skeletal systems). In this review, the bi-directional regulation between cells from immune and skeletal systems is highlighted, with particular emphasis given to how this interaction affects the process and balance of bone remodelling. Then, the direct effects of S1P-S1PR1 signalling on osteoclastogenesis, osteogenesis, and osteoclast-osteoblast coupling is illustrated. The role of this signalling in immune modulation is explained, as well as the possible impacts of these modulations on bone remodelling. Finally, the significance of the S1P-S1PR1 signalling in destructive bone diseases, and the available therapeutic approaches based on this signalling are evaluated.

In *Chapter 3*, the status of S1P-S1PR1 signalling was examined and found to be abnormally activated in the apical periodontitis lesions. The animal model further confirmed the activation of S1P-S1PR1 signalling and its possible relation with over-activated osteoclastogenesis. This relationship was verified by modulation of the S1P-S1PR1 signalling to reduce bone loss. Taken together, it was found that the S1P-S1PR1 signalling was induced during infection-stimulated inflammation, and was actively involved in the pathogenesis of bone loss by enhancing osteoclastogenesis.

In *Chapter 4*, the interaction between macrophages and bone marrow-derived mesenchymal stromal cells (BMSCs) was considered to be involved in the activation of S1P-S1PR1 signalling during infections. To uncover how this activation happens, an *in vitro* co-culture system of macrophages and BMSCs was stimulated with lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria which triggers a strong immune response in mammals, in order to mimic infection. Under these circumstances, the secreted factors derived from macrophages were found to induce the S1P production of BMSCs, which acted in an autocrine manner to activate S1PR1 in BMSCs. S1PR1 was found to result in enhanced RANKL (an indispensable factor in osteogenesis) production in BMSCs. In this Chapter, the interaction between macrophages and BMSCs, when exposed to LPS, resulted in the activation of S1P-S1PR1 signalling in BMSCs, which eventually induced osteoclastogenesis by stimulating RANKL expression.

In *Chapter 5*, the possible impacts of S1P-S1PR1 signalling on osteogenesis during infection-induced inflammation were investigated. Osteogenic markers were up-regulated in BMSCs co-cultured with macrophages, especially under the infection-induced low inflammatory condition. The expression of osteogenic markers was reduced when S1PR1 was blocked in BMSCs in the co-culture system, which indicated that S1P-S1PR1 has an essential role in promoting osteogenesis during low infection-induced inflammation.

Chapter 6 summarized the findings and discussed future perspectives based on what are the limitations of this study.

Chapter 2: Literature Review

Suggested Statement of Contribution of Co-Authors for Chapter by Published Paper

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Lan Xiao	Involved in the concept and design of the project, wrote the manuscript.
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Thor Friis	Assisted with manuscript preparation.
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Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

Name: Prof Yin Xiao Signature: 

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S1P-S1PR1 signalling: the "Sphinx" in osteoimmunology

2.1 ABSTRACT

Bone remodelling is strictly regulated by immune modulators to maintain the balance between bone resorption and formation. This fundamental interaction between the immune and skeletal systems is described as osteoimmunology, a process achieved through a number of modulators. Among these modulators is the pleiotropic sphingolipid metabolite, S1P, which together with its cognate receptors, the S1PRs, are a known regulators of the immune system, which are also directly involved in the process of bone remodelling. S1P-S1PR1 signalling is considered essential for osteoimmunology, being a pathway capable of inducing an inflammatory response that stimulates osteoclastogenesis, as well as osteogenesis. Its role in bone remodelling is far from straight forward, especially under pathological conditions, such as inflammation. In this review, we highlight the dual role of S1P-S1PR1 signalling in bone remodelling and immune-modulation with an emphasizing on how osteo-immune regulators are affected by inflammation, an issue with relevance to conditions such as rheumatoid arthritis and periodontitis.

2.2 INTRODUCTION

It has been long realized that the immune and skeletal systems are closely linked. The process of bone remodelling is a primary process required for bone homeostasis and consists of osteoclasts-driven bone resorption and osteoblasts-driven bone formation (Raggatt & Partridge, 2010). The immune progenitor cells-derived osteoclasts and mesenchymal stem cells-derived osteoblasts are linked *via* immune modulators and are, therefore, the fundamental cell types of these two interconnected systems. This relationship was first termed osteoimmunology to describe the interaction of cells from the immune and skeletal systems (Arron & Choi, 2000). Further studies within the realm of osteoimmunology have revealed that a complex system of mutual regulation exists between cells from the immune and skeletal systems. At one level, the immune response greatly affects osteoclast-osteoblast coupling and therefore mediates the balance between bone resorption and formation, whereas on another level, cells from skeletal system also have a profound effect on the differentiation and function of immune cells.

Sphingosine-1-phosphate (S1P) is one of the most important sphingolipid metabolites and has a number of roles that are essential in a diverse range of cellular processes during both physiological and pathophysiological conditions (Maceyka, et al., 2012). S1P is produced by various cell types and acts not only as an intracellular second messenger, but also an extracellular first messenger in both an autocrine and paracrine manner. It does this by binding with a class of G-protein-coupled receptors, known as sphingosine-1-phosphate receptors, of which there is currently five known subtypes, S1PR1 through to S1PR5, (Spiegel & Milstien, 2011). Of these receptors, S1PR1 is expressed in most types of mammalian cells (Aarthi, Darendeliler, & Pushparaj, 2011) and considered to be multifunctional in many biological processes. The S1P-S1PR1 signalling has long been addressed as a key regulator in immune response due to its involvement in the chemotaxis, activation, differentiation and function of immune cells (Rivera, et al., 2008). The elevated concentration of S1P, coupled with an up-regulation of S1PR1 expression locally within inflammatory tissues in many diseases, as well as the therapeutic effects of S1PR1 modulators, is an indication of the important role of S1P-S1PR1 signalling in inflammation (Maceyka, et al., 2012).

S1P-S1PR1 signalling is considered to be a catalyst of inflammation by inducing osteoclastogenesis whilst reducing osteogenesis; however, the fact that this pathway is also active during bone regeneration (Pederson, et al., 2008; Sato, et al., 2012) suggests a complex and rather intriguing role in bone remodelling. This review attempts to highlight the interactions between the immune and skeletal systems, how these interactions effect bone remodelling, and what is known about the role of S1P-S1PR1 signalling in the emerging field of osteoimmunology.

2.3 BONE REMODELLING & OSTEOIMMUNOLOGY

2.3.1 Bone biology

Bone is the main constituent of the vertebrate skeletal system and supports and protects a number of organs in the body (Steele & Bramblett, 1988). Bone plays an essential role in calcium metabolism, acting as the main repository of calcium, and also acts as reservoir of growth factors and cytokines. Bone marrow is a crucial organ,

not only for the capacity of haematopoiesis and fat storage, but also for its role as an important immune organ where immune cells develop and differentiate (Taichman, 2005). The major cellular components of bone are osteoclasts, osteoblasts, and osteocytes. Besides cells, bone also consists of collagen and osteoid, as well as inorganic mineral deposits (Kini & Nandeesh, 2012).

2.3.2 Bone remodelling

The skeleton undergoes a life-long and continuous process of remodelling, in which old bone is resorbed by osteoclasts and new bone replaced by osteoblasts (Raggatt & Partridge, 2010). Nearly 10% of the skeletal mass in an adult human is remodelled annually (Takayanagi, 2007). This dynamic bone turnover reshapes the skeleton during growth, maintains calcium homeostasis, repairs micro damages, and enables bone to adapt to different biomechanical forces. Bone remodelling, therefore, plays a key role in maintaining its strength and integrity (Hadjidakis & Androulakis, 2006).

Osteoclasts and osteoblasts are the major players in the bone remodelling process. The hematopoietic stem cells (HSCs)-derived osteoclasts are considered the only cells capable of resorbing bone (Kini & Nandeesh, 2012). Osteoclast precursors fuse with each other to form a giant, multinucleated cell—the osteoclast (Boyle, Simonet, & Lacey, 2003). Osteoclastogenesis depends on receptor activator of nuclear factor-kappa B ligand (RANKL), a cytokine in the tumour necrosis factor (TNF) family (Lacey et al., 1998). RANKL activates its cognate receptor, receptor activator of nuclear factor-kappa B (RANK), initiating osteoclastogenic signals (Fig 1). The RANKL-RANK axis, together with the downstream NF-κB signalling pathway, is indispensable in osteoclastogenesis (Kong et al., 1999; Theill, Boyle, & Penninger, 2002). Another key factor in osteoclast formation is macrophage colony-stimulating factor (M-CSF), which is critical in regulating survival and proliferation of osteoclast precursors (Yoshida et al., 1990). M-CSF also up-regulates RANK expression in osteoclast precursors, making the cells more sensitive to RANKL, thereby contributing to RANKL-RANK-derived osteoclastogenesis (Ross & Teitelbaum, 2005).

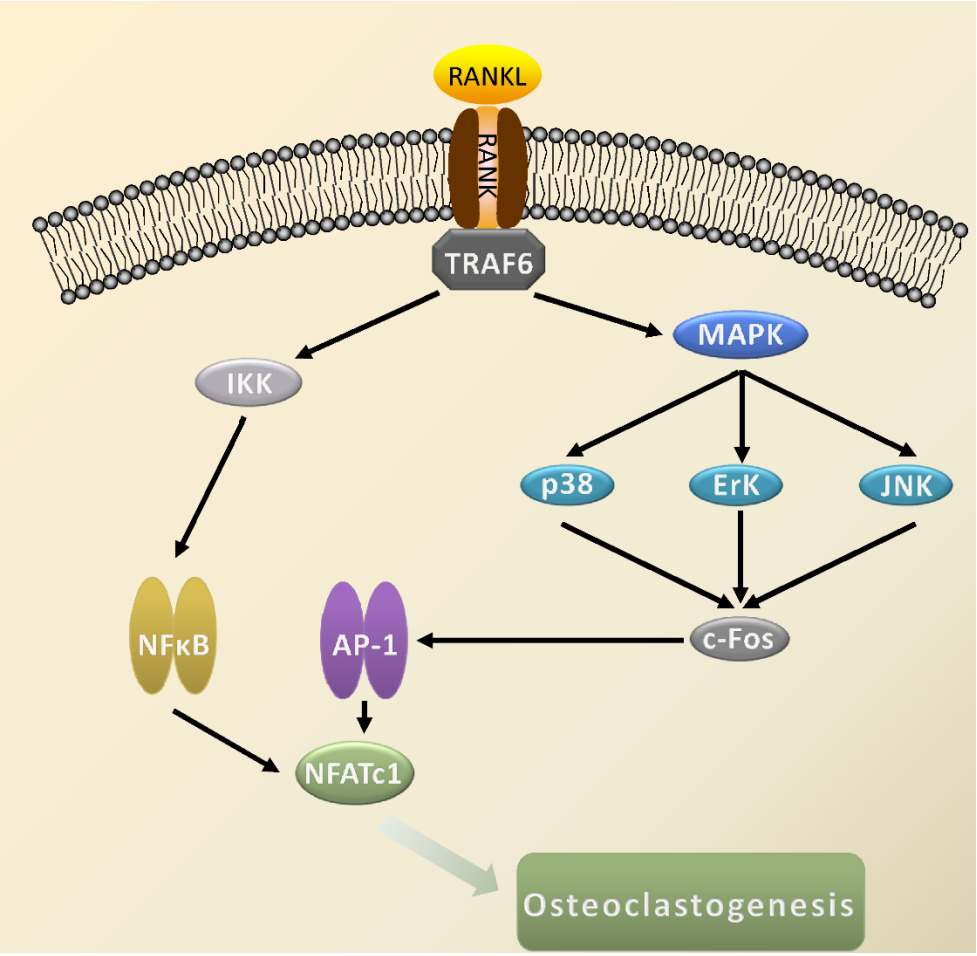


Figure 1. The RANKL-RANK axis mediated osteoclastogenic signals. RANK is activated when combining with its ligand RANKL. Activated RANK then triggers the down-stream osteoclastogenic signalling cascades. Activated TRAF6 induces the MAPK, IKK and NF-κB signalling, which eventually result in activation of NFATc1 and osteoclastogenesis. RANKL: receptor activator of nuclear factor factor-kappa B ligand. RANK: receptor activator of nuclear factor-kappa B. TRAF6: tumour-necrosis factor (TNF) receptor-associated factor 6. IKK: inhibitor of nuclear factor kappa-B kinase. MAPK: mitogen-activated protein kinase. NF-κB: nuclear factor kappa B. AP-1: activator protein1. ErK: extracellular signal regulated kinase. JNK: c-Jun N-terminal kinase. NFATc1: nuclear factor of activated T-cells, cytoplasmic 1.

Osteoclastogenesis interacts with osteoblasts–the major producer of RANKL and M-CSF (Udagawa et al., 1990)–indicating that osteoclasts and osteoblasts are related "coupling" cells that link osteoclastogenesis to osteogenesis. Osteoblasts are derived from MSCs and are the main cell type responsible for bone formation (Kini & Nandeesh, 2012). The typical markers of osteogenesis include early stage markers such as alkaline phosphatase (ALP), type I collagen (COL1), runt-related transcription factor 2 (RUNX2), bone sialoprotein (BSP), and late stage markers such as osteopontin

(OPN) and osteocalcin (OCN). The Wnt/β-catenin and TGF-β signalling pathways, as well as the signal transducer and activator of transcription 3 (STAT3) signalling, are also considered to be crucial in the process of bone formation (Logan & Nusse, 2004; O'Brien, Gubrij, Lin, Saylors, & Manolagas, 1999; Urist, 1965). Besides RANKL and M-CSF, osteoblasts also produce osteoprotegerin (OPG), which, conversely, plays a "protective" role in bone by acting as a decoy receptor of RANKL and interrupts its binding to RANK, thereby impeding osteoclastogenesis (Simonet et al., 1997). Hence, osteoclasts and osteoblasts are interconnected by the RANKL/RANK/OPG axis, with the ratio of RANKL to OPG determining the balance between bone resorption and formation (Fonseca et al., 2004).

Under normal physiological conditions, bone remodelling is a strictly regulated process that must maintain bone formation at a rate equal to that of bone resorption (Arron & Choi, 2000). Skeletal pathologies arise when this balance is disrupted. The most common of such disorders is when bone remodelling is skewed towards resorption–that is, when osteoclastogenesis is aberrantly stimulated so the rate of bone resorption exceeds bone formation, resulting in a net bone loss, as seen in inflammatory diseases, such as rheumatoid arthritis (RA) (Rodan & Martin, 2000), periodontitis (Taubman, et al., 2005), and apical periodontitis (Wang & Stashenko, 1993). Hence, studying the mechanisms of physiological/pathological remodelling could help us find therapeutic approaches against these diseases.

2.3.3 Osteoimmunology

Evidence of the relationship between the immune and skeletal systems became apparent with the finding that IL-1, secreted by antigen-stimulated immune cells, played a positive role in osteoclastogenesis (Dewhirst, Stashenko, Mole, & Tsurumachi, 1985). Since then, many more studies have demonstrated the role of immune system on bone remodelling (Fig 2) (Takayanagi, 2007). Furthermore, cells derived from skeletal system, such as MSCs, are capable of regulating immune response (Nauta & Fibbe, 2007). Such findings gave birth to osteoimmunology, a field that is concerned with cellular and molecular interactions between immune and skeletal systems.

Regulations of the immune system on bone remodeling

Classic examples of osteoimmunology are inflammatory bone diseases, such as RA, in which a dysregulated immune response causes excessive osteoclastogenesis and bone destruction (Firestein, 2003). The adaptive immune cells–T-helper cells–play a critical role by producing RANKL, the key factor in osteoclastogenesis, and also produce other factors that regulate bone remodelling. Cytokines derived from type 1 helper T (Th1) cells, such as IFNγ and granulocyte-macrophage colony-stimulating factor (GM-CSF), suppress osteoclastogenesis by interrupting the RANK signalling (Fig 1) (Kim, Day, & Morrison, 2005; Lari et al., 2007; Miyamoto et al., 2001; Takayanagi et al., 2000). Other cytokines derived from type 2 helper T (Th2) cells, such as interleukin-4 (IL-4) and IL-10, also inhibit RANK signalling and osteoclast differentiation (Abu-Amer, 2001; Moreno, Kaczmarek, Keegan, & Tondravi, 2003; Park-Min et al., 2009). IL-6, which is produced by Th2 cells and M1 macrophages, induces osteoclastogenesis by promoting RANKL production, as well as stimulating IL-1 production, which amplifies the inflammatory response (Hashizume, Hayakawa, & Mihara, 2008; Kudo et al., 2003; Kurihara, Bertolini, Suda, Akiyama, & Roodman, 1990). IL-6 also induces the differentiation of type 17 helper T (Th17) cells, which secrete the pro-inflammatory cytokine IL-17 (Kimura, Naka, & Kishimoto, 2007; Korn et al., 2008), which in turn promote RANKL secretion and osteoclastogenesis (Lubberts et al., 2003; Van Den Berg & Miossec, 2009)

The immune-suppressive regulatory T (Treg) cells(Sakaguchi, Yamaguchi, Nomura, & Ono, 2008), inhibit osteoclastogenesis in a direct cell-to-cell contact-dependent manner, by binding of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) on Treg cells with CD80 and CD86 on osteoclast precursors; Treg cells also reduce osteoclastogenesis by secreting IL-4 and IL-10 (Zaiss et al., 2007). Another Treg cell-derived factor, TGF-β, has pleiotropic effects on osteoclastogenesis. On one hand, TGF-β can induce osteoclast differentiation by promoting RANK expression and regulate activator protein 1 (AP-1) signalling (Galvin, Gatlin, Horn, & Fuson, 1999; Quinn et al., 2001), a key downstream effector of RANK (Fig 1). However, in osteoclast-osteoblast co-cultures, TGF-β can also suppress RANKL expression in osteoblasts, effectively applying the breaks on osteoclastogenesis (Quinn, et al., 2001).

Cells from the innate immune system also contribute to the regulation of osteoclastogenesis. Macrophages, the major components of innate immunity,

constitute three sub-populations of cells: (1) non-activated M0 macrophages; (2) pro-inflammatory M1 macrophages, which are classically activated by LPS or Th1 cell cytokines such as IFNγ; and (3) M2 macrophages, which is alternatively activated by Th2 cell cytokines, such as IL-4 or IL-13, and are classified as anti-inflammatory macrophages (Horwood, 2015; Mantovani et al., 2004; Mills, Kincaid, Alt, Heilman, & Hill, 2000; Murray et al., 2014). The M1 macrophages express IL-1α and IL-1β, TNF-α, nitric oxide (NO), and reactive oxygen species (ROS), whereas M2 macrophages are characterized by the expression of IL-10, TGF-β, and arginase-1 (Arg1) (Ho & Sly, 2009; Mantovani, et al., 2004; Tjiu et al., 2009). Macrophages are precursors of osteoclasts (Takeshita, Kaji, & Kudo, 2000) and secrete factors that actively affect osteoclastogenesis. The M1 macrophages express IL-1α and IL-1β which activates RANK signalling thereby inducing osteoclastogenesis, under both physiological and pathological conditions (Wei, Kitaura, Zhou, Ross, & Teitelbaum, 2005; Zwerina et al., 2007). M1 macrophages also express TNF-α, which stimulates osteoclast differentiation by activating the NF-κB signalling (Kobayashi et al., 2000; Lam et al., 2000). In osteoblasts, TNF-α promotes RANKL expression, which also affects osteoclastogenesis (Kitaura et al., 2004; Zou, Hakim, Tschoep, Endres, & Bar‐Shavit, 2001). Whereas M1 macrophages secrete high levels of IL-12 and low levels of IL-10, M2 macrophages do the opposite: secreting high levels of IL-10 and low levels of IL-12 (Ho & Sly, 2009; Mantovani, et al., 2004; Tjiu, et al., 2009). IL-10 is a negative regulator of osteoclastogenesis (Park-Min, et al., 2009) and IL-12 suppresses osteoclast differentiation, by inducing the expression of IFNγ and GM-CSF, both of which downregulate RANK signalling (Nagata, Kitaura, Yoshida, & Nakayama, 2003).

Compared with its role in osteoclastogenesis, the role of immune system in osteogenesis is not as clear cut. It has been reported that pro-inflammatory T cells and IFNγ can promote osteogenesis (Croes et al., 2016), whereas a mouse knockout study has shown that *interferon gamma receptor 1* (*IFNGR1*) knockout resulted in lower bone density and inferior osteogenic capacity of MSCs (Duque et al., 2009), indicative of a positive role for IFNγ in osteogenesis; however, T cells and IFNγ can inhibit *in vivo* osteogenesis by downregulating RUNX2 (Liu et al., 2011). IL-17, another pro-inflammatory cytokine with a seemingly contradictory role in osteogenesis, has been shown to enhance ALP activity and induce bone formation *in vitro* and *in vivo* (Huang

et al., 2009; Ono et al., 2016), but also inhibit osteogenesis in a calvarial defect model in rat (Kim et al., 2014). Treg cell-derived TGF-β has been identified as an activator of osteogenesis by promoting osteoblast differentiation and enhanced mineralization (Bonewald & Dallas, 1994).

There are also conflicting results from studies of macrophage-derived cytokines. The M1-derived cytokines, IL-1 and TNF-α, have been identified as negative regulators of *in vitro* osteogenesis, since they impede the ALP activity and mRNA expression of *ALP, COL1A1, RUNX2, OCN* and *OPN* (Nanes, 2003; Perrien et al., 2002). However, IL-1 is reported to induce *in vitro* osteogenesis *via* the Wnt-5a/receptor tyrosine kinase–like orphan receptor 2 (Wnt-5a/Ror2) pathway ; moreover, *in vivo* bone formation is inhibited following antagonism of IL-1 receptor, suggesting IL-1 is required in osteogenesis (Ma et al., 2003; Sonomoto et al., 2012). It has also been suggested that TNF-α can facilitate bone formation, in spite of its suppression of RUNX2 and COL1, by enhancing the expression and activity of ALP and the expression of bone morphogenetic protein 2 (BMP-2) *via* the NF-κB pathway, (Hess, Ushmorov, Fiedler, Brenner, & Wirth, 2009). Conflicting results such as these indicate that the roles of IL-1 and TNF-α in osteogenesis remain unresolved. The conflict results might be due to the different doses and duration of stimulation of these factors. The opposite results between *in vitro* and *in vivo* studies imply that except for MSCs, other cells such as immune cells also take indispensable roles in *in vivo* osteogenesis; for this reason, it might be inaccurate to simply investigate the effect of some certain factors on MSCs *in vitro*, instead, more efforts should be put into the research on the interplay between MSCs and immune cells during osteogenesis.

IL-6, which is classified as an M1 cytokine, can also enhance *in vivo* ALP activity *via* the immune signal mediator STAT3, a further indication of the ability of IL-6 to affect osteogenesis (Bellido, Borba, Roberson, & Manolagas, 1997; Blanchard, Duplomb, Baud'huin, & Brounais, 2009; Cho et al., 2007; Itoh et al., 2006; Sammons, Ahmed, El-Sheemy, & Hassan, 2004). Oncostatin M (OSM) is another M1 inflammatory cytokine that acts *via* STAT3 and has a role in osteogenesis where it serves as a coupling factor between pre-osteoclasts and pre-osteoblasts by activating RUNX2. Studies with OSM or OSM receptor (OSMR) deficient mice show reduced bone healing, evidence for its role in osteogenesis (Guihard et al., 2012; Song, Jeon, Kim, Jung, & Kim, 2007). Besides secreting osteogenic factors, macrophages also

recruit MSCs. For example, the Osteoactivin (OA)/Glycoprotein non-metastatic melanoma protein B (GPNMB), a transmembrane glycoprotein mainly secreted by M2 macrophages, is a chemotactic agent that stimulates the migration of MSCs (Wu, Sondag, Malcuit, Kim, & Safadi, 2015).

In fractures, the acute inflammatory phase–characterized by the infiltration of M1 macrophages–is indispensable for bone healing. Secreted factors of M1 macrophages, especially OSM can induce significant osteogenesis, whereas conditioned media (CM) from M2 macrophages does not elicit such a strong osteogenic effect despite being associated with tissue repair (Guihard, et al., 2012). The effect on osteogenic differentiation of pre-osteoblasts by direct co-culture with M0, M1, or M2 macrophages show that with any one of these will enhance osteogenic potential. Furthermore, the conversion of M1 to M2 macrophages significantly improves mineralization in the co-culture system (Loi et al., 2016). These similar studies all support the indispensable role of macrophages in osteogenesis. It could be presumed that the transient activation of M1 macrophages is essential for the early osteoblast activation, while M2 macrophages is indispensable for the later mineralization. It is now thought that inflammatory signals at the injury site serve as chemoattractant that causes macrophages to migrate to the defect. Once in place, the macrophages proliferate, polarize, and release functional factors, which then recruit MSCs to the injury site and induce bone formation (Takayanagi, 2007).

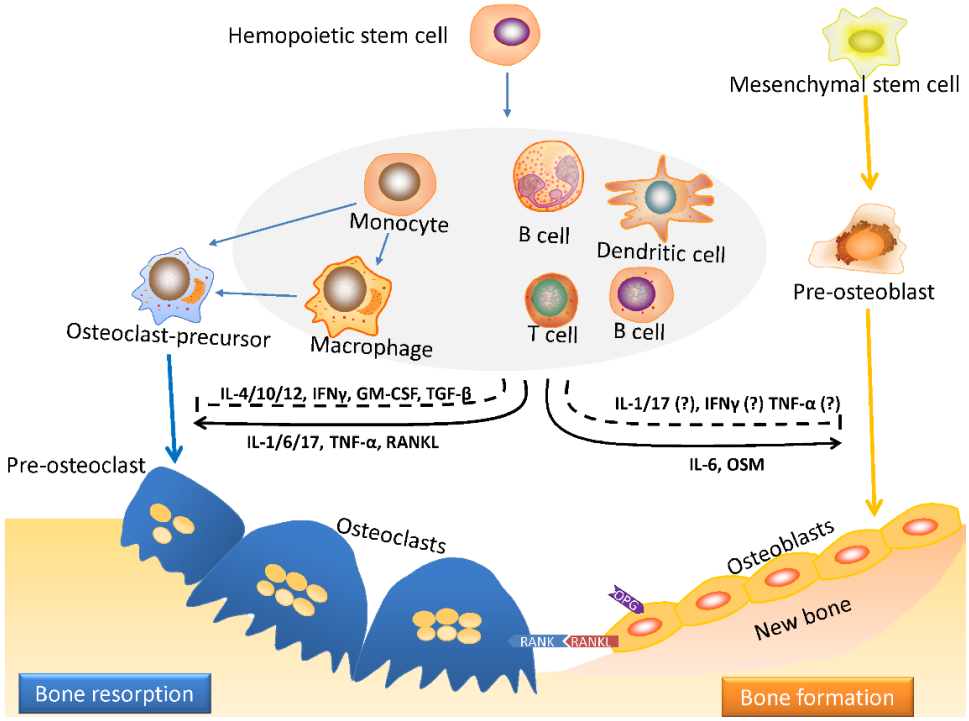


Figure 2. The immuno-regulation on bone remodelling. The two major players in bone remodelling – osteoclasts and osteoblasts are coupled through the RANKL-RANK-OPG axis, that osteoblasts-derived RANKL combines with its ligand RANK in osteoclasts and plays an indispensable role in osteoclastogenesis; OPG (which is also derived from osteoblasts) reduces osteoclastogenesis by impairing the RANKL-RANK signalling. The immune system greatly takes part in osteoclastogenesis by producing RANKL; also, the immune-related factors either affect pre-osteoclasts, or interacts with osteoblasts to induce RANKL production to regulate osteoclastogenesis. On the other hand, the immuo-regulators also affect the process of osteogenesis. RANKL: receptor activator of nuclear factor factor-kappa B ligand. RANK: receptor activator of nuclear factor-kappa B. OPG: osteoprotegerin.

Immune-regulation mediated by cells from the skeletal system

Skeletal system also exerts a regulatory effect on immune system. It is well recognised that MSCs, the non-hematopoietic multipotent stem cells that play a central role in osteogenesis, are capable of immunomodulation. MSCs are able to suppress the differentiation and function of cells from both innate and adaptive immune system, with the exception of Treg cells. MSCs are reported to affect macrophage polarization by inducing the M2 subsets; interfere with T cell proliferation, cytokine production and polarization, especially inducing the Treg cells differentiation whereas reducing that of Th17 cells. The similar reduction has also been found in the activation of dendritic cells (DCs), B cells and NK cells (Aggarwal & Pittenger, 2005; Fibbe, Nauta, & Roelofs, 2007; Gur-Wahnon, Borovsky, Beyth, Liebergall, & Rachmilewitz, 2007; Nauta & Fibbe, 2007; Németh et al., 2009). These immune-suppressing functions of MSCs are achieved either through direct cell-cell contact or secretion of soluble immune-modulators, some of which are produced constantly while others are produced when MSCs are exposed to inflammatory factors or activated immune cells (Bunnell, Betancourt, & Sullivan, 2010). The direct cell-cell contact suppression is achieved through the programmed death 1 (PD-1) pathway (Augello et al., 2005). The immune suppressive factors include prostaglandin E2 (PGE2), TGF-β, IL-10, indoleamine 2,3-dioxygenase (IDO), human leukocyte antigen G (HLA-G), hepatocyte growth factor (HGF), leukemia inhibitory factor (LIF), IL-1 receptor antagonist (IL-1RA), monocyte chemotactic protein 1 (MCP1), galectin-1, galectin-3, semaphorin-3A, TNF-α-induced protein 6 (TNAIP6) (Auletta, Deans, & Bartholomew, 2012; Bunnell, et al., 2010).

When toll-like receptors (TLRs) are activated by LPS, IFN-α/γ, or TNF-α, MSCs may exert immune-suppressive effects such as converting M1-like macrophages to M2-like macrophages by secreting PGE2 (Chen et al., 2010; Maggini et al., 2010;

Németh, et al., 2009). However, MSCs can also respond by producing pro-inflammatory cytokines (Bunnell, et al., 2010), such as IL-1β and IL-6, or chemokine IL-8, which attract the migration of neutrophils and augment inflammatory response (Raicevic et al., 2010). It has emerged that similar to macrophages, human MSCs also polarizes into two distinct phenotypes: pro-inflammatory MSC1 and immunosuppressive MSC2 (Waterman, Tomchuck, Henkle, & Betancourt, 2010). TLR signalling plays an active role in this polarization, in which acute and low-level activation of TLR4 directs MSCs toward MSC1 phenotype, whereas TLR3 activation induces an MSC2 phenotype. MSC1 phenotype can also be induced by IFNs or direct contact with certain pro-inflammatory cells. Polarized MSCs are thought to play roles similar to that of M1 and M2 macrophages in tissue repair (Verreck, de Boer, Langenberg, van der Zanden, & Ottenhoff, 2006), with MSC1s contributing to early stage inflammation and MSC2s contributing to late tissue regeneration.

Based on these findings, it is speculated that during physiological bone healing, the inflammatory signals at the injury site attract macrophages to migrate to the defect by means of a chemotactic response. Once in place, the macrophages polarize towards M1 phenotype and release functional factors, which play an indispensable role in the initial stage of bone formation by recruiting MSCs to the injury site and inducing osteogenesis. On the other hand, the MSCs-derived immune-suppression gradually induces the transition from M1 to M2 phenotypes. The importance of this transition implies that M2 macrophages might play an indispensable role in the later stage of bone formation. The critical mutual regulation between macrophages and osteoblasts/pre-osteoblasts should take a great part in osteogenesis; hence, studying on this interaction should be meaningful to bone regeneration.

Over all, the skeletal and immune systems, which share a variety of regulatory molecules and mechanisms, exert mutual regulation of each other, and this interaction eventually maintains the physiological status of body. Cells from each system exert regulatory functions to maintain the homeostasis of bone. In addition, studies into the factors that are involved in the interplay between these two systems should give us a better understanding of osteoimmunology. Among these factors, the multifunctional sphingolipid metabolites-S1P, together with its receptors (i.e. S1PR1), not only acts as a key regulator in immune-regulation, but also directly affects the process of bone

remodelling, therefore, making the S1P-S1PR1 signalling essential for osteoimmunology.

2.4 S1P-S1PR1 SIGNALLING & OSTEOIMMUNOLOGY

2.4.1 The function of S1P and its receptor S1PR1

Sphingolipid is a key component of mammalian cell membranes and undergo metabolism in response to certain stimulations (Rivera, et al., 2008). The sphingolipid is first cleaved to produce ceramide, which is then deacylated by ceramidases (CERase) to produce sphingosine. Phosphorylation of sphingosine forms the multifunctional bioactive lipid S1P, a mediator of a number of cellular processes, such as cell proliferation, survival, differentiation, migration, as well as cytokine and chemokine production (Spiegel & Milstien, 2011). The phosphorylation of sphingosine is based on sphingosine kinase 1 and 2 (SPHK1 and SPHK2). S1P can be reversibly degraded by S1P phosphatases (SPPs), or irreversibly degraded by S1P lyase (SPL) (Hannun & Obeid, 2008; Mechtcheriakova et al., 2007; Peest et al., 2008). SPHK1 is mainly present in the cytoplasm which, after being activated by certain stimuli, is translocated to the cell membrane where it can catalyse the phosphoration of sphingosine and result in the production of S1P (Pitson, 2011). SPHK1 is activated by factors such as TNF-α and IL-1β, as well as LPS. This activation was found to be dependent on extracellular signal regulated kinase (Erk) signalling (Billich et al., 2005; Hammad et al., 2008; Xia et al., 1998). Whereas SPHK1 is mainly responsible for the production of secreted S1P, SPHK2 induces the nuclear S1P production and, therefore, plays a role in the regulation of gene transcription (Hait et al., 2009).

After its synthesis, S1P is readily degraded by SPL and SPPs in most mammalian cells, except in erythrocytes, which lack SPL and SPPs (Rosen, Gonzalez-Cabrera, Sanna, & Brown, 2009). Platelets also produced copious amounts of S1P due to an absence of SPL (Ito et al., 2007). This explains the discrepancy, under normal physiological conditions, between circulating S1P levels in the blood versus that of solid tissues, with S1P concentration being significantly higher in peripheral blood (micro-molar range) than in solid tissues. S1P is also maintained at relatively high level (greater than 100 nano-molars) in the lymph circulation, which is mainly due to

the presence of lymphatic endothelial cells (Pappu et al., 2007; Schwab et al., 2005; Venkataraman et al., 2008). Cells from the macrophage–monocyte lineage are also recognized as important producers of S1P (Ryu et al., 2006). Especially, under inflammatory conditions, SPHK1 is abnormally activated and produces high levels of S1P that is released into the local microenvironment. TNF-α also induces SPHK1 led production of S1P in human umbilical vein endothelial cells (HUVEC) and L929 fibroblasts (Pettus et al., 2003; Xia, et al., 1998). Other inflammatory factors such as IL-1β and IFN-γ have also been shown to activate SPHK1 (Alvarez, Milstien, & Spiegel, 2007; Billich, et al., 2005), which partially explains the high S1P levels in the inflammatory tissues (Ledgerwood et al., 2008). Furthermore, the vascular leakage accompanied by inflammation may release S1P from blood to tissues, thereby raising the S1P concentrations within the inflammatory tissues (Kono & Proia, 2015).

Intracellular S1P is considered a second messenger, which contributes to the suppression of apoptosis (Spiegel & Milstien, 2003), whereas the extracellular S1P regulates pleiotropic biological functions by binding with its cognate G-protein-coupled receptors in an autocrine or/and paracrine manner (Sanchez & Hla, 2004). To date, the five known S1P receptors couple with diverse heterotrimeric G-protein subunits (known as Gαi, Gαq/11 and Gα12/13) following activation, thereby directing many diverse signalling pathways (Davis & Kehrl, 2009). Of these receptors, S1PR1 is considered to be most widely expressed and found in most tissues, especially the lungs, brain, and immune organs (Cahalan et al., 2011; Chae, Proia, & Hla, 2004; Kono et al., 2014). S1PR1 is expressed by a variety of cells, and following activation S1P, interacts with Gαi which then activates the downstream signalling molecules (Fig 3), such as phospholipase C (PLC), phosphoinositide 3-kinase (PI3K), Ras guanosine triphosphatase (GTPase) and Rac GTPase. These molecules then subsequently activate their downstream signalling pathways, including NF-κB, p38 mitogen-activated protein kinase (MAPK), Erk, Akt, and mammalian target of rapamycin (mTOR) (Melendez et al., 2007; Nayak et al., 2010; Takabe, Paugh, Milstien, & Spiegel, 2008).

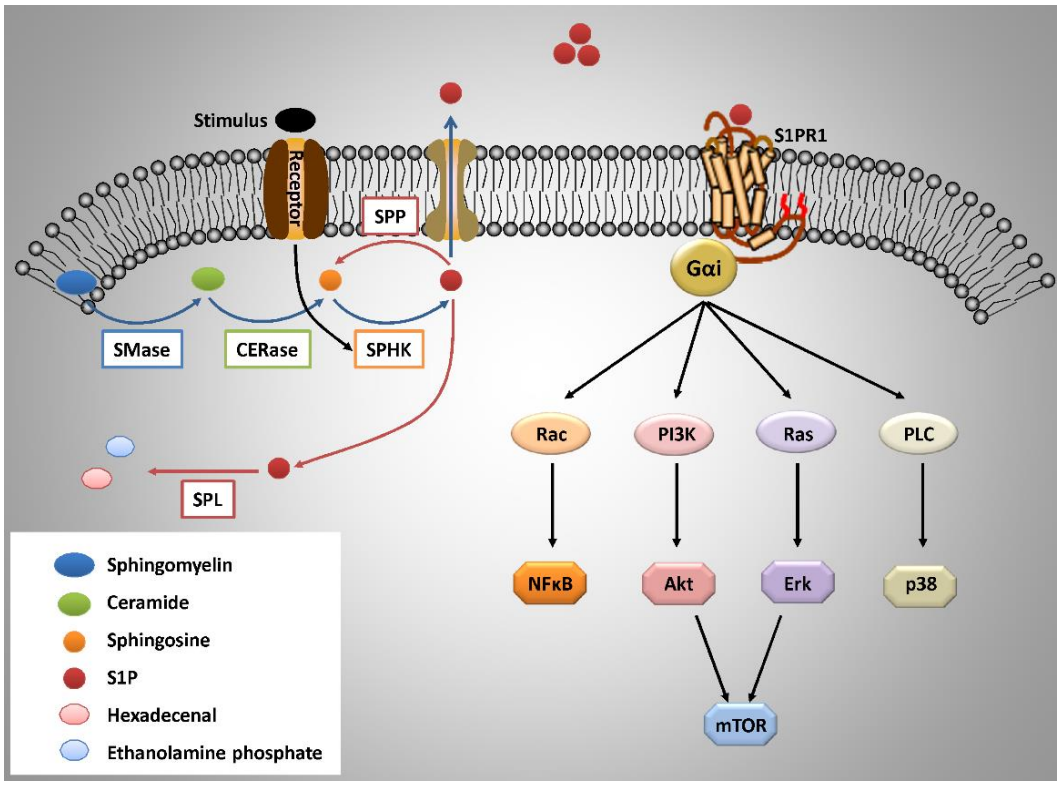


Figure 3. The S1P-S1PR1 signalling. Sphingolipid (derived from cell membrane) is cleaved (by sphingomyelinases, SMase) to ceramide. Ceramide is then deacylated by ceramidases (CERase) to produce sphingosine. S1P is produced by phosphorylation of sphingosine, which is mediated by SPHKs (SPHK1 and SPHK2, which can be activated by certain stimulus). S1P can be reversibly degraded by S1P phosphatases (SPPs), or irreversibly degraded by S1P lyase (SPL). On the other hand, S1P can be transported outside the cells and acts in the autocrine or paracrine manners to activate its receptor S1PR1. The S1PR1 then activates its down-stream signal cascades and therefore regulates diverse cell activities. S1P: Sphingosine-1-phosphate. S1PR1: Sphingosine-1-phosphate receptor 1. PLC: phospholipase C. PI3K: phosphoinositide 3-kinase. NF-κB: nuclear factor kappa B. ErK: extracellular signal regulated kinase. mTOR: mammalian target of rapamycin.

S1PR1 is considered a key player in the development of the vascular system and is highly expressed in differentiating endothelial cells (Liu et al., 2000). S1PR1 is required to maintain the integrity of endothelial cell barrier and thus regulates vascular permeability responses, especially under inflammatory conditions (Camerer et al., 2009). SPHK1 is induced by inflammation and enhances S1P production of endothelial cells, which then acts in a feed-forward manner to stimulate more S1PR1 expression, counteracting the increased permeability caused by pro-inflammatory mediators e.g., LPS, thereby preventing otherwise lethal cell-leakage in response to inflammation. In

epithelial cells, S1PR1 take part in the maintenance of epithelial cell barrier integrity, which also initiates the immune defence against the invading pathogens (Eskan et al., 2008). S1PR1 is expressed in MSCs and acts as a key regulator in cell migration, proliferation, differentiation, and survival (Pitson & Pébay, 2009), whereas in osteoclast- and osteoblast-precursor cells S1PR1 expression is associated with their differentiation (Ishii et al., 2009; Sato, et al., 2012), further testament to its role in bone remodelling.

2.4.2 Role of the S1P-S1PR1 signalling in bone remodelling

S1P has been found to induce both osteoclastogenesis and osteogenesis, a dual role that makes S1P-S1PR1 signalling all the more intriguing.

S1P-S1PR1 signalling in osteoclastogenesis

Together with its ligand S1P, S1PR1 directs chemotactic migration of osteoclast precursors *in vitro* and *in vivo*, thus having a directly involvement in osteoclastogenesis. S1P-S1PR1 signalling is thought to regulate osteoclast precursors trafficking to and from the bone surface, where the precursor cells fuse and differentiate into osteoclasts, which dynamically regulate bone mineral homeostasis and osteoclastogenesis (Ishii, et al., 2009). S1PR1-dependent chemo-attraction is only activated when S1P concentration is comparably low – high concentrations of S1P activates another receptor, S1PR2 on the precursor cells and triggers an S1PR2-dependent chemo repulsion (Ishii, Kikuta, Shimazu, Meier-Schellersheim, & Germain, 2010). This mechanism partially explains how these precursor cells are retained in the bone marrow, which has lower levels of S1P than in the peripheral blood. S1PR1 and S1PR2 act in a concerted manner to regulate osteoclast precursors egressing from bone marrow into circulation, depending on the relative concentrations of S1P. During RANKL-mediated osteoclast differentiation, the activity of SPHK1 (the catalyst for S1P synthesis) is significantly enhanced, resulting in increased production of S1P by the precursor cells. Conversely, inhibition of SPHK1 leads to suppression of osteoclastogenesis (Ryu, et al., 2006).

S1P-S1PR1 signalling in osteogenesis

Although S1P is considered an osteoclastogenic factor, it also plays a positive role in osteogenesis. In the process of BMP-2-mediated osteoblast differentiation, S1P

significantly induces ALP activity and the expressions of key bone formation markers, such as OCN and RUNX2. Enhanced BMP-2/Smad signalling is the result of MEK1/2-Erk1/2 pathway activation (Sato, et al., 2012). Conditioned medium from osteoclasts can induce osteogenesis and is thought to be due to Wnt10b, BMP-6 and S1P secreted into the medium. And whereas S1P and BMP-6 can trigger the migration of pre-osteoblasts towards bone resorption sites, S1P can also induced osteogenic differentiation of the same cells by activating S1PR1, a finding that became apparent when S1PR1 was blocked (Pederson, et al., 2008). These properties of S1P-S1PR1 signalling go some way in explaining how bone formation is initiated following bone resorption.

S1P-S1PR1 signalling in osteoclast-osteoblast coupling

Interestingly, S1P, which is produced during osteoclastogenesis, also inhibits this process, by supressing p38-MAPK signalling, a key signalling pathway downstream of RANK (Fig 1). This suggests S1P targets cells other than osteoclasts, e.g., the coupling osteoblasts (Ryu, et al., 2006). S1P binds S1PR1–3 on osteoblasts which activates p38-MAPK and Erk signalling, resulting in increased levels of cyclooxygenase-2 (COX2). COX2 induces the expression of prostaglandin E2 (PGE2), which prompts the production of RANKL by osteoblasts. RANKL binds to its receptor RANK on the osteoclast precursors which promotes osteoclast differentiation and S1P secretion, thereby setting up a feedforward loop for osteoclastogenesis.

Cathepsin K (CSTK) is an enzyme that is involved in bone degradation which, when specifically deleted in osteoclast lineage by targeted *in vivo* gene modification, results in mild osteopetrosis, a condition characterized by an increased number of osteoblasts and bone formation, as well as an increased number of dysfunctional osteoclasts and impaired bone resorption (Lotinun et al., 2013). *In vitro* analysis of primary osteoblasts showed enhanced ALP activity and osteogenic potential, as well as increased RANKL/OPG ratio. The osteoclasts of *CSTK*-knockout mice presented with up-regulated expression of SPHK1 and increased S1P production. The increased RANKL/OPG ratio of the primary osteoblasts explains the increased number of osteoclasts. The antagonist of S1PR1 and S1PR3 reduced the osteogenic ability of osteoblasts induced by the conditioned medium of CSTK-knock out osteoclasts, suggests the induced *in vivo* osteogenesis is due to the activation of S1PR1 and S1PR3 (Lotinun, et al., 2013).

2.4.3 S1P-S1PR1 signalling and osteoimmunology

S1P-S1PR1 signalling also regulates bone remodelling indirectly by modulating the immune response. S1PR1 is as a key regulator of a diverse range of immune cell activities, which when binding with S1P, leads to cell migration, proliferation, and differentiation (Rivera, et al., 2008). The key role of S1P-S1PR1 signalling in regulating the trafficking and egression of immune cells has been identified in both the innate and adaptive immune systems, affecting cells such as HSCs, DCs, macrophages (monocytes), neutrophils, mast cells, T and B lymphocytes, natural killer T (NKT) cells (Cinamon, Zachariah, Lam, Foss, & Cyster, 2008; Ishii, et al., 2009; Jenne et al., 2009; Jolly et al., 2004; König et al., 2010; Rathinasamy, Czeloth, Pabst, Förster, & Bernhardt, 2010; Schwab & Cyster, 2007; Wang et al., 1999; Zachariah & Cyster, 2010). S1P-S1PR1 signalling is required for mature thymocytes to egress from the thymus, and for T/B cells to egress from secondary lymphoid tissues into blood or lymph under homeostatic and pathological conditions (Allende et al., 2010; Sinha, Park, Hwang, Davis, & Kehrl, 2009; Zachariah & Cyster, 2010; Zhang et al., 2012). Lymphocytes with decreased S1PR1 expression have an impaired ability to egress. Furthermore, S1PR1 deficiency results in blocked lymphocytes egression, a condition known as lymphopenia (Sinha, et al., 2009). This suggests a vital role of S1P-S1PR1 signalling in directing the timely and appropriate distribution of immune cells in lymphatic and non-lymphatic tissues which aids homeostasis of the immune system. During inflammation, there is a spike of the local concentration of S1P, which activates S1PR1 and leads to the recruitment of the immune cells, such as effector T cells, to the inflamed tissues and their *in situ* retention (Ledgerwood, et al., 2008).

S1P-S1PR1 signalling is also a key modulator of immune cell differentiation and function. S1PR1 is required for the maturation of DCs. The functions of mature DCs are also regulated by S1PR1, which further affects the activation and polarization of T cells (Czeloth et al., 2007; Maeda et al., 2007). The lymphocyte responses are greatly affected by S1P. For example, S1P-S1PR1 signalling regulates the function and especially the polarization of $CD4^+$T cell subsets. S1PR1 activation in $CD4^+$T cells impairs the production of Th1 cells-derived IFNγ, while enhancing the production of Th2 cells-derived effector cytokine IL-4, thereby downregulating the Th1 cell response while upregulating Th2 cells (Dorsam et al., 2003; Gräler, Huang, Watson, & Goetzl, 2005; Song et al., 2008; Wang, Huang, & Goetzl, 2007). On the other hand,

S1P was found to induce the differentiation and activities of pro-inflammatory Th17 cells, as well as the production of IL-17 (the main effector cytokine of Th17 cells) *in vitro*, accompanied by reduced production of Th1 and Th2 cells-derived cytokines, a process that is considered to be S1PR1-dependent (Huang, Watson, Liao, & Goetzl, 2007; Liao, Huang, & Goetzl, 2007). Of note, signalling through S1PR1 impedes the differentiation and function of Treg cells, a vital regulator in immune response due to the special immune-suppressive activities, by activating the downstream Akt-mTOR signalling pathway, thereby promoting the immune response (Liu et al., 2009; Liu, Yang, Burns, Shrestha, & Chi, 2010).

However, in macrophage polarization, S1P-S1PR1 signalling favours the differentiation of an anti-inflammatory phenotype, inducing a conversion of the pro-inflammatory M1 macrophage subset to the anti-inflammatory M2 subset; S1PR1 is required for this conversion (Hughes et al., 2008). The S1P-derived induction of Th2 response and IL-4 secretion may indirectly affect this process. Therefore, in contrast to its immune-inductive role in $CD4^+$ T cell polarization, S1P-S1PR1 signalling has an immune-suppressive role in determining macrophage polarization, which altogether complicates its role in inflammation.

S1P-S1PR1 signalling participates in bone remodelling by way of immunomodulation. As a key factor in immune cells migration (Thangada et al., 2010), S1P-S1PR1 signalling induces the recruitment of activated immune cells in the inflammatory tissue and therefore promotes the inflammatory response, a process that induces bone resorption (Takayanagi, 2007). As mentioned above, S1P-S1PR1 signalling promotes Th17 cells differentiation and IL-17 production (Liao, et al., 2007), both of which promote osteoclastogenesis (Sato et al., 2006), and which impedes the differentiation and function of Treg cells (Liu, et al., 2009; Liu, et al., 2010), thereby further facilitate immune response and osteoclastogenesis (Sakaguchi, et al., 2008; Zaiss, et al., 2007). More importantly, S1P enhanced RANKL production of $CD4^+$T cells and hence greatly inducing osteoclast differentiation (Takeshita et al., 2012). However, S1P-S1PR1 signalling might also act as a negative factor in osteoclastogenesis, as it polarizes macrophage differentiation from M1 towards the M2 subsets (Hughes, et al., 2008), which is thought to reduce osteoclast differentiation. This conversion from M1 to M2 phenotype is driven by S1P-S1PR1 signalling and may also take part in osteogenesis. M1 macrophage activation, it has been suggested,

is indispensable during the early stages of bone repair. M2 macrophages, on the other hand, plays a key role in the later stage of bone formation, suggesting the shift from M1 to M2 phenotype is a crucial process in the regulation of osteogenesis (Guihard, et al., 2012; Loi, et al., 2016).

Taken together, these studies all point to S1P-S1PR1 signalling having a key role in osteoimmunology. Whereas at one level it directly regulates osteoclastogenesis, osteogenesis, and osteoclast-osteoblast coupling and, therefore, has a crucial role in bone remodelling; however, at another level, the immune regulation of S1P-S1PR1 signalling suggests an indirect modulation of osteoclastogenesis and osteogenesis. Under certain pathological conditions, the aberrant status of S1P-S1PR1 signalling results in dysregulation of immune response and consequently imbalanced bone remodelling.

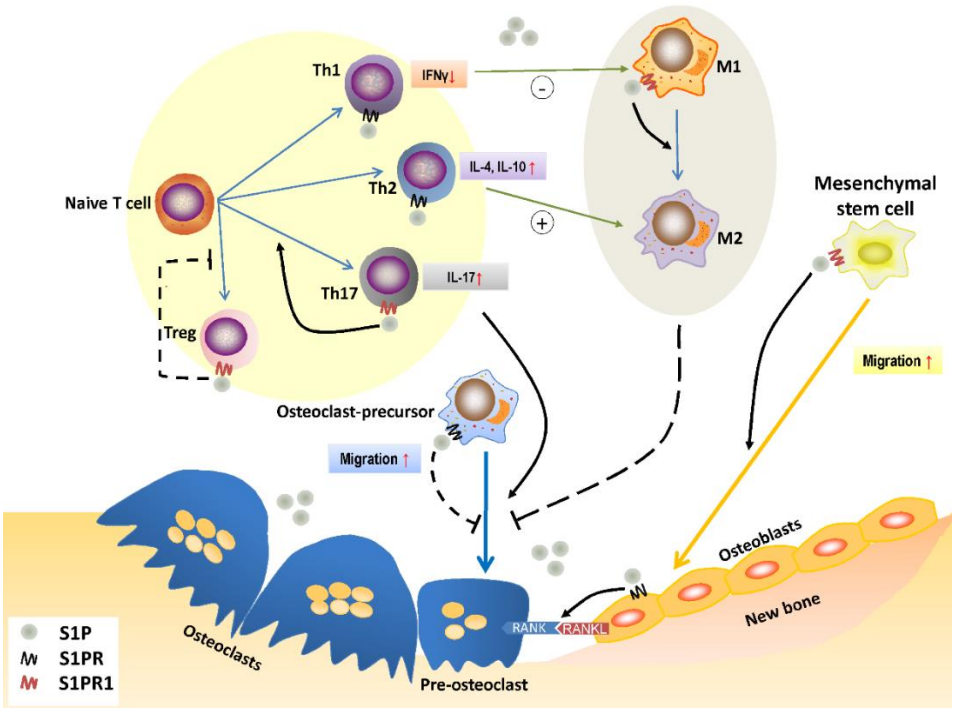


Figure 4. The role of S1P-S1PR1 signalling in osteoimmunology. S1P-S1PR1 signalling is greatly involved in the interaction between immune system and bone remodelling. On one hand, S1PR1 directly affects osteoclastogenesis by inducing the migration of osteoclast-precursors. The direct effect of S1P on osteoclast-precursors results in reduced osteoclastogenesis; however, it induces RANKL production of osteoblasts and facilitating the RANKL-RANK mediated osteoclastogenesis. S1P also induces the migration of MSCs and osteogenesis by activating S1PR1. On the other hand, S1P-S1PR1 signalling participates in immune regulation, which affects the polarization and function of T-helper cells. S1P-S1PR1 signalling induces the differentiation and function of Th17 cells (known as inducing osteoclastogenesis) while impedes that of Treg cells (known as reducing osteoclastogenesis); therefore facilitating osteoclastogenesis. S1P also induces the function of Th2 cells while reduces that of Th1 cells, which affects the macrophage phenotype; also, S1P directly induces the transition of M1 to M2 phenotype by activating S1PR1. This conversion of pro-inflammatory M1 macrophages to tissue-engineering M2 macrophages therefore impedes osteoclastogenesis, which might also affect osteogenesis.

2.5 S1P-S1PR1 SIGNALLING IN BONE DESTRUCTIVE DISEASES

Abnormally activated S1P-S1PR1 signalling has been observed in many diseases, such as rheumatoid arthritis (RA), multiple sclerosis and cancer, and is thought to have a key role in the pathogenesis of these diseases (Choi et al., 2011; Feng et al., 2010; Kitano, et al., 2006). The importance of S1P-S1PR1 signalling in immune regulation and bone remodelling makes it necessary to assess their putative roles in the pathogenesis of bone destructive diseases.

2.5.1 S1P-S1PR1 signalling in rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disorder that feature excessive osteoclastogenesis resulting from an abnormally activated immune response (Kitano, et al., 2006). Abnormal expression of S1PR1 in inflamed RA synovial tissue and S1P-SIPR1 signalling has been shown to play an important role in the pathogenesis of RA in the mechanism of promoting the COX-2-induced PGE2 secretion by rheumatoid synoviocytes (which was identified as a key process in RA-related osteoclastogenesis) (Kitano, et al., 2006). S1P-S1PR1 signalling has been found to promote RANKL production in $CD4^{+}$T cells and synoviocytes *in vitro* following stimulation with TNF-α, suggesting a role of S1P-S1PR1 signalling in RA bone destruction (Takeshita, et al., 2012). Therapies targeting S1P or S1PR1 have shown some promise as a treatment for RA and further indication of the key role of S1P-S1PR1 signalling in this disease. Fingolimod, also known as FTY720, is an analogue of sphingosine and acts as a 'functional antagonist' of S1PR1 *in vivo* by inducing the internalization, down-regulation and degradation of S1PR1 (Pham, Okada, Matloubian, Lo, & Cyster, 2008; Thangada, et al., 2010). FTY720 has been shown to be effective in a mouse RA model by inhibiting the infiltration of effector $CD4^{+}$ T cells. This reduced the production of PGE2 by the synoviocytes and induced the Th2 cells-mediated immune response (Tsunemi et al., 2010). S1PR1 is therefore a potential therapeutic target in RA. In a mouse TNF-α induced chronic inflammatory arthritis model, the progression and severity of arthritis, as well as bone destruction in mouse with SPHK1 deficiency were significantly reduced. This was associated with reduced COX-2 expression and Th17 differentiation caused by decreased S1P production, with a resulting inhibition of osteoclastogenesis in the inflammatory joints (Baker, Barth, Chang, Obeid, &

Gilkeson, 2010)–a finding that suggests SPHK1 and/or S1P can also be potential targets for TNF-α-related arthritis, such as RA.

2.5.2 S1P-S1PR1 signalling in infection-induced bone loss

Since the S1P-S1PR1 signalling plays such an important role in the immune response it is not surprising that this pathway involved in a diverse range of infections caused by microbial agents such as viruses, bacteria and fungi (Arish, et al., 2015). Although S1P-S1PR1 signalling was found to be up-regulated in most systemic inflammations, its status in infection-induced inflammations has been determined by the types of pathogens involved. A common target of pathogens is SPHK1. Pathogens can either induce or reduce SPHK1 activity, resulting in dysregulated S1P expression. This hinders the normal immune response and creates suitable conditions for pathogen evasion. In some cases pathogens can promote S1P signalling in the infected cells and prolong the survival of these cells *via* the activation of the PI3K/Akt and Erk signalling pathways, which then facilitates the replication of pathogens (Monick et al., 2004). However, in some cases pathogens can suppress S1P production thereby impairing the innate and adaptive immune response against the invading pathogen. For example, in *Mycobacterium tuberculosis (M. tuberculosis*) infections, S1P signalling is disrupted by bacteria-mediated reduction of SPHK1 activity. Clinical treatment of S1P results in significantly reduced intracellular growth of *M. tuberculosis*, as well as enhanced antigen presentation and $CD4^+$ T cell response (Malik et al., 2003; Richmond, Lee, Green, Kornfeld, & Cruikshank, 2012). The way that different pathogens have a differential effect on S1P expression is an example of the complexities implicit in the infection-induced inflammatory response. Therefore, a thorough understanding of the S1P-S1PR1 signalling status might shed some light on the management and treatment of the infection-induced inflammatory diseases.

S1P is also associated with the pathogenesis of periodontitis, an inflammatory oral bone-destructive disease caused by bacterial infection (Yu, Sun, & Argraves, 2016). In a mouse periodontitis model, the ablation of SPHK1 significantly attenuated alveolar bone loss and is accompanied by reduction in the numbers of leukocytes and osteoclasts in the periodontal tissues, suggesting modulating S1P production could be a novel therapeutic strategy for periodontitis treatment. It could be presumed that the

S1P-S1PR1 signalling also takes part in other infection-induced bone destructive diseases, such as apical periodontitis and osteomyelitis.

Taken together, the importance of S1P-S1PR1 signalling in bone-destructive diseases further confirmed the vital role of this signalling in bone remodelling, suggesting this signalling should be considered as a new therapeutic approach for these diseases. Also, the role of S1P-S1PR1 signalling in other infection-induced bone-destructive diseases (i.e. apical periodontitis) deserves future study.

2.6 CONCLUSION

In summary, the literature is replete with in vitro and in vivo studies that indicate that S1P acts as a key coupling factor between osteoclasts and osteoblasts, thereby playing a key role in immune regulation and bone remodelling via the activation of S1PR1. The S1P-S1PR1 signalling pathway must be considered as a crucial factor in osteoimmunology and, therefore, a novel therapeutic target for the treatment of diseases with inflammatory bone-destruction. Furthermore, the detailed mechanism of S1P-S1PR1 signalling in bone remodelling under inflammation is still not clear and needs further research.

Chapter 3:

Suggested Statement of Contribution of Co-Authors for Chapter by Published Paper

In the case of this chapter

Title: **Different correlation of sphingosine-1-phosphate receptor 1 with receptor activator of nuclear factor kappa B ligand and regulatory T cells in rat periapical lesions (Xiao et al., 2015)**

Date, status, journal: April 2015, Published, Journal of Endodontics

Contributor	Statement of contribution
Lan Xiao	Involved in the conception and design of the project, performed laboratory experiments, data analysis and interpretation. Wrote the manuscript.
Yinghong Zhou	Involved in the conception and design of the project, data analysis and reviewed the manuscript.
Rong Huang	Assisted with data analysis.
Wei Shi	Assisted with sample collection and preparation.
Bin Peng	Involved in the conception and design of the project, and reviewed the manuscript.
Yin Xiao	Involved in the conception and design of the project, and reviewed the manuscript.

Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

Name: Prof Yin Xiao Signature: 

Date: 27th July 2016

The role of S1P-S1PR1 signalling in infection-induced inflammatory bone loss

3.1 ABSTRACT

Background: The S1P-S1PR1 signalling is crucial for the regulation of immunity and bone metabolism. This study aims to investigate the status of S1P-S1PR1 signalling in apical periodontitis lesions and its role in the infection-induced inflammatory bone loss *in vivo*.

Methods: Periapical lesion samples from 10 patients were collected and examined by histological observation, immunohistochemistry, and double immunofluorescence analysis. Apical periodontitis model was created in rats to further investigate the correlation between S1P-S1PR1 signalling and the development of the lesion in a time sequence. Five rats were sacrificed on day 0, 7, 14, 21, 28, and 35, and their mandibles were harvested. X-ray imaging, micro-computed tomography (μCT) scanning, histological observation, immunohistochemistry, enzyme histochemistry, and double immunofluorescence analysis were performed on the harvested samples. To demonstrate the effect of signalling modulation on the disease progress, Fingolimod (FTY720; S1P-S1PR1 signalling modulator) administration was performed on experimental group while saline vehicle was applied as control. Five rats in each group were sacrificed on day 7, 14, 21, 28, and 35, respectively. The mandibles were harvested and subjected to the mentioned analysis for comparison.

Results: The S1P-S1PR1 signalling was up-regulated in both human and rat periapical lesions. A correlation was found between the expressions of S1PR1 and RANKL; the $S1PR1^+$ and $RANKL^+$ cells were found in the inflammatory periapical tissues. S1P-S1PR1 signalling was negatively modulated by FTY720, which effectively inhibited bone resorption and osteoclastogenesis by down-regulating RANKL production in the periapical lesions.

Conclusions: The S1P-S1PR1 signalling was abnormally up-regulated in apical periodontitis lesion, which enhanced RANKL production and induced osteoclastogenesis, thereby promoting bone loss in the lesions. The S1P-S1PR1 signalling could be a potent target for the treatment of apical periodontitis.

3.2 INTRODUCTION

Apical periodontitis is one of the most common inflammatory dental diseases. Mainly due to bacterial infection of (tooth) root canals, this disease usually results in pulp damage, lesion formation around the apex of the root, as well as alveolar bone destruction (Garlet et al., 2012; Stashenko, Teles, & D'Souza, 1998). The lesion represents the inflammatory immune response to pathogen invasion, including infiltration and activation of immune cells, induced production of inflammatory molecules, which then promote activation of osteoclastogenesis and therefore resulting imbalanced bone remodelling (which consists of bone resorption and formation) (Stashenko, Yu, & Wang, 1992).

Osteoclasts are known as the only cells conducting bone resorption (Kini & Nandeesh, 2012). Previous studies have identified that the differentiation and function of osteoclasts are regulated by immune system-derived factors, such as immune cells, cytokines, receptors, and transcription factors (Takayanagi, 2007). Receptor activator of nuclear factor kappa B ligand (RANKL) – a tumour necrosis factor family cytokine– is identified to be indispensable in osteoclastogenesis (Yasuda et al., 1999). On the other hand, osteoprotegerin (OPG), a number of the TNF receptor superfamily, acts as a decoy receptor for RANKL and therefore impairs RANKL-mediated osteoclastogenesis (Simonet, et al., 1997). The balance between RANKL and OPG is considered as the hallmark in determination of the balanced bone remodelling (Fonseca, et al., 2004). In the pathogenesis of apical periodontitis, increased RANKL expression has been found to be associated with osteoclast formation during periapical bone destruction (Zhang & Peng, 2005). Furthermore, the imbalance between RANKL and OPG has been identified to play a crucial role in induced apical periodontitis of rats (Kawashima et al., 2007). Among the immune cells, activated T cells are considered to be crucial during the immune process of apical periodontitis, not only for their role in maintaining a balanced immune defence against oral bacteria, but also for their ability to produce RANKL under inflammatory circumstances and thereby inducing osteoclastogenesis (Matsuo, Ebisu, Shimabukuro, Ohtake, & Okada, 1992; Theill, et al., 2002).

Sphingosine-1-phosphate receptor 1 (S1PR1) is one of the five members of the endothelial differentiation gene (Edg) family of G protein-coupled receptors that are expressed at the cellular membrane (Chi, 2011). Its ligand, sphingosine-1-phosphate

(S1P) is a multifunctional bioactive lipid molecule, which binds with S1PR1 to mediate diverse cellular process, such as cell growth, survival, differentiation, migration, and cytokine production (Spiegel & Milstien, 2011). S1PR1 is expressed in most immune cells, the S1P-S1PR1 signalling plays a central role in regulating immune response, including cell movement, differentiation and functional maturation, under both physical and pathological circumstances (Rivera, et al., 2008; Spiegel & Milstien, 2011). S1P-S1PR1 signalling is also involved in directing the migration of osteoclast precursors; moreover, S1P is reported to induce RANKL production in activated T cells, which suggests that this particular signalling cue may act as a regulator in osteoclastogenesis and is therefore important in bone resorption (Ishii, et al., 2009; Takeshita, et al., 2012). Abnormally enhanced S1P-S1PR1 signalling has been identified in inflammatory bone destructive disease such as rheumatoid arthritis, leading to the speculation that this signalling plays a key role in the pathogenesis of inflammatory diseases (Kitano, et al., 2006). It has also been reported that S1P production is dysregulated in various infectious diseases (by up-/down-regulation of SPHK1). This dysregulation impairs the normal status of immune response and therefore creating a suitable circumstance for the survival and evasion of the pathogens (Arish, et al., 2015). Hence, the role of S1P-S1PR1 signalling in the pathogenesis of apical periodontitis – a special infection induced bone destructive disease–should be further elucidated.

Fingolimod (FTY720) has been applied clinically as immune response modulator for the treatment of multiple sclerosis (MS). FTY720 has been proved to be effective in animal models of autoimmune diseases (Fujino et al., 2003; Kappos et al., 2006; Rausch et al., 2004; Sanford, 2014). Reportedly, FTY720, which is an analogous to S1P, induces internalization and degradation of S1PR1 on lymphocytes and makes S1PR1 unresponsive to S1P, hence, inhibiting the S1P-S1PR1 signalling-dependent immune response (Pham, et al., 2008; Thangada, et al., 2010). Based on this, it is considered that FTY720 is a negative modulator for S1P-S1PR1 signalling.

This study was aimed to investigate the role of S1P-S1PR1 signalling in the pathological development of apical periodontitis. The S1P-S1PR1 signalling was found to be activated in both human and rat periapical lesions. Furthermore, a strong positive correlation was found in the expression of S1PR1 and RANKL; moreover, modulating the S1P-S1PR1 signalling was found to be effective in inhibiting the bone

loss derived from apical periodontitis, which was partially achieved by reducing RANKL-mediated osteoclastogenensis. Therefore, the S1P-S1PR1 signalling plays a crucial role in the pathogenesis of apical periodontitis.

3.3 MATERIALS AND METHODS

3.3.1 Patients and sample collection

All experimental procedures were approved by the Institutional Review Board of the Ethics Committee, School and Hospital of Stomatology, Wuhan University, China. Periapical lesions (total n = 10) were collected from patients diagnosed with apical periodontitis during tooth extraction at the Department of Oral and Maxillofacial Surgery, School and Hospital of Stomatology, Wuhan University, Wuhan, China. The patients (aged from 20 to 68 years) were free of systemic diseases and had not taken antibiotics for at least 1 month. The periapical tissues obtained from 10 patients underwent tooth extraction for orthodontic treatments were used as control samples (the teeth were healthy with no sign of inflammation in the periodontal tissues). The collected samples were fixed with 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS, pH 7.4) for 24 h at room temperature. After that they were dehydrated and embedded in paraffin, then cut into 4 μm-thick histologic sections.

3.3.2 Induction of rat apical periodontitis model

All experimental procedures conform to the Guiding Principles of the Animal Care and Use Committee in the School of Stomatology, Wuhan University. This experiment was performed as previously described (Stashenko, Wang, Tani-Ishii, & Yu, 1994). Thirty female Wistar rats, each weighing approximately 200 g, were purchased from the Experimental Animal Centre of Hubei Province. After being randomly divided into six groups, apical periodontitis models were induced by exposure of the dental pulp of the first lower molars under anaesthesia (intraperitoneal administration of ethyl urethane (0.8 mg/g)); a #1/4 round bur was used to expose the pulp while avoiding damages of the pulp-chamber floor. Cavities were left open to the oral environment during the whole procedure. Five rats from each group were sacrificed in 0, 7, 14, 21, 28, and 35 days after the operation. Day 0 group served as the negative control. The mandibles were harvested for further analysis.

3.3.3 FTY720 administration to the induced apical periodontitis lesions

All experimental procedures conform to the Guiding Principles of the Animal Care and Use Committee in the School of Stomatology, Wuhan University. Fifty

female Wistar rats, each weighing approximately 200 g, were purchased from the Experimental Animal Centre of Hubei Province. Periapical lesions were induced as described above. The rats were randomly and equally divided into two groups: control group and experimental group. Each group received injections beginning at two days after the periapical lesion induction and continued every two days during the entire experimental period. For the experimental group, intragastric administration of FTY720 (LC Laboratories, Woburn, USA) was applied at a dose of 3 mg/kg (diluted by saline) body weight every two days, whereas only saline (the vehicle) was injected in the control group. Five rats from each group were sacrificed at 7, 14, 21, 28, and 35 days after the operation. The mandibles were harvested for further analysis.

3.3.4 Sample preparation

The periapical tissues were fixed with 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS, pH 7.4) for 24 h at room temperature. After that they were dehydrated in graded ethanol (70%, 90%, and 100%), cleared in xylene, embedded in paraffin, and then cut into 4 μm-thick histologic sections for H & E staining, Immunohistochemistry staining and Immunofluorescence staining. One in every four sections was analysed by light microscopy.

The mandibles were fixed with 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS, pH 7.4) for 24 h at room temperature. Samples were firstly photographed under X-ray (DXS PRO, BRUKER) and then scanned by μCT (samples were placed in suitable containers filled with 0.5% paraformaldehyde during scanning). All mandibles were then demineralized in 10% EDTA solution at room temperature for 4 to 5 weeks. The decalcified samples were then dehydrated and embedded in paraffin. Finally, the mandibles were cut into 4 μm frontal serial sections in the mesiodistal direction. The section selected for staining (H & E staining, immunohistochemistry staining and immunofluorescence staining) should contain the distal root of the first mandibular molar, as well as the apex of root canal. One in every four stained sections was analysed by microscopy.

3.3.5 High-resolution X-ray imaging and μCT analysis

High resolution X-ray images were taken and analysed by the BRUKER Molecular Imaging software to assess the bone intensity around periapical lesions. Bone intensity of the light blue area around the distal root apex was considered to be relatively low. Measurement of this area was conducted using the software Image-Pro Plus 6.0. The mandibles were scanned with a Scan μCT 50 imaging system (Scanco Medical, Bassers-dorf, Switzerland) at a resolution of 20 μm, and then reconstructed to evaluate the bone loss around the distal root of the first molar. The raw data were analysed by the three-dimensional (3D) image analysis software (VGStudio MAX, Heidelberg, Germany) to measure the volume of the periapical region as previously described (Yang et al., 2014). All measurements were performed in a double blind manner by two trained independent observers.

3.3.6 Histological analysis

Sections were first dewaxed in xylene, then rehydrated in graded ethanol (100%, 90%, and 70%). After rehydration, the sections were stained with hematoxylin for 2 min, rinsed with tap water for 5 min and then dipped 3 times in acid ethanol. After dehydration, they were stained with eosin for 15 sec, rinsed in 100% ethanol twice, cleared in xylene, and then mounted with mounting medium. One in every four sections was analysed by light microscopy (Leica, Wetzlar, Germany). The areas of periapical lesions (the tissue around the lower third of the roots) were measured by two trained independent observers using the software Image-Pro Plus 6.0. Three sections of each mandible were evaluated, and the mean average was calculated to measure the size of the periapical lesion in each mandible. All measurements were performed in a double-blind manner by two trained independent observers.

3.3.7 Tartrate-resistant Acid Phosphatase (TRAP) assay

TRAP staining was performed to detect osteoclasts. All procedures were performed following the manufacturer's instructions (Acid Phosphatase, Leukocyte (TRAP) Kit, Sigma-Aldrich, St. Louis, MO). Briefly, sections were dewaxed in xylene, rehydrated in graded ethanol (100%, 90%, and 70%), and washed by distilled water. Sections were then incubated in a solution of naphthol AS-BI phosphoric acid and Fast

Garnet GBC for 1 h at 37 °C. Sections incubated in a substrate-free medium served as TRAP controls. After incubation, sections were lightly counterstained with hematoxylin (15 sec), cleared in xylene, and then mounted with mounting medium by cover slips. The TRAP-positive cells, which coloured from dark red to purple, were identified as osteoclasts if they presented with two or more nuclei.

3.3.8 Immunohistochemistry

Immunohistochemical staining was performed as previously described (Yang, et al., 2014). Briefly, sections were dewaxed in xylene, then rehydrated in graded ethanol (100%, 90%, and 70%), and washed by distilled water. For antigen retrieval, pepsin (rat samples, Maixin, Fuzhou, China) were applied to the sections for 15 min. Sections were then blocked with 2.5% bovine serum albumin (diluted in PBS) for 1 h. Rabbit polyclonal antibodies against S1P (Abcam, Cambridge, UK), rabbit polyclonal antibodies against S1PR1 (Santa Cruz Biotechnology, Santa Cruz, CA), goat polyclonal antibodies against human RANKL and OPG (Santa Cruz Biotechnology, Santa Cruz, CA) at dilutions of 1:400, 1:100, 1:200 and 1:150, respectively, were used as primary antibodies. All the antibodies were diluted in PBS containing 0.1% BSA. Sections for negative controls were substituted with non-immune rabbit or goat serum instead of primary antibodies. After incubated with primary antibodies at 4 ℃ overnight, sections were stained with the SP kit (Maixin, Fuzhou, China) (for S1P and S1PR1) and PV kit (Maixin, Fuzhou, China) (for RANKL and OPG) according to the manufacturer's instructions. After that, the sections were developed with diaminobenzidine (DAB) and then counterstained with hematoxylin, cleared in xylene, and mounted with cover slips. In each section, osteoclasts/S1P-positive cells/S1PR1-positive cells/RANKL-positive cells/OPG-positive cells in five randomly selected regions within periapical tissue (the tissue around lower 1/3 part of the roots) were counted under 400× magnification by two independent observers. The average number of the five chosen areas was calculated. Three sections from each mandible were analysed for cell measurements. All measurements were performed in a double-blind manner by two trained independent observers.

3.3.9 Double immunofluorescence labelling

This experiment was performed as previously described (Liu & Peng, 2013). Sections were dewaxed in xylene, rehydrated in graded ethanol (100%, 90%, and 70%), and washed by distilled water. Antigen retrieval was performed by incubation with pepsin for 15 min at 37 ℃. After that, they were incubated with 2.5% bovine serum albumin (BSA, diluted in PBS) for 1 h and then incubated overnight with S1PR1 antibodies (human samples: 1:25; rat samples: 1:25) and RANKL antibodies (human samples: 1: 100; rat samples: 1:75) at 4 °C. The sections were then washed and incubated with the secondary fluorescent antibodies, donkey anti-rabbit Dylight488 (EarthOx, San Francisco, CA, USA) (1:100) and donkey anti-goat-CY3 (Proteintech Group, Wuhan, China) antibodies (1:250) at for 1 h. All antibodies were diluted in 1% BSA/PBS. Nuclei were stained with 4', 6-diamidino-2-phenylindole. Finally, the sections were washed, incubated with AutoFluo Quencher (Applygen, Beijing, China) for 30 min, mounted and viewed under fluorescence microscope (Leica, Wetzlar, Germany).

3.3.10 Statistical analysis

All data were subjected to statistical analysis using one-way ANOVA, followed by the Student-Newman-Keul test at a = 0.05. Pearson correlation was used for correlation analysis on numbers of $S1PR1^+$ cells with osteoclasts and $RANKL^+$ cells ($\alpha = 0.05$). A $p < 0.05$ was considered to significantly different. Data were analysed by SPSS 13.0 (SPSS Inc., Chicago, IL). All data were presented as mean ± standard deviation (SD).

3.4 RESULTS

3.4.1 Up-regulated S1P-S1PR1 signalling and RANKL expression in human periapical lesions

In this study, firstly, general morphology of human lesion/normal periapical tissues was examined by histological observation. As shown in Fig 5, the periapical lesion was analogous to granulomatous tissue, which consisted of fibroblast-like cells and infiltrating immune cells; hardly any epithelial cells were observed. The control

tissue showed the appearance of normal periodontal ligament tissue, which mainly consisted of fibroblasts; no obvious immune cell infiltration was observed.

The status of S1P-S1PR1 signalling in human periapical lesion tissues was examined by IHC staining. As shown in Fig 5, compared with the control tissue, the S1P expression was more intensive in the periapical lesions. Furthermore, S1PR1 expression was found to be up-regulated (Fig 5). It could also be observed that the expression of RANKL was enhanced in the lesions (Fig 5), suggesting the cells in the lesion were responsible for the induced osteoclastogenesis in apical periodontitis.

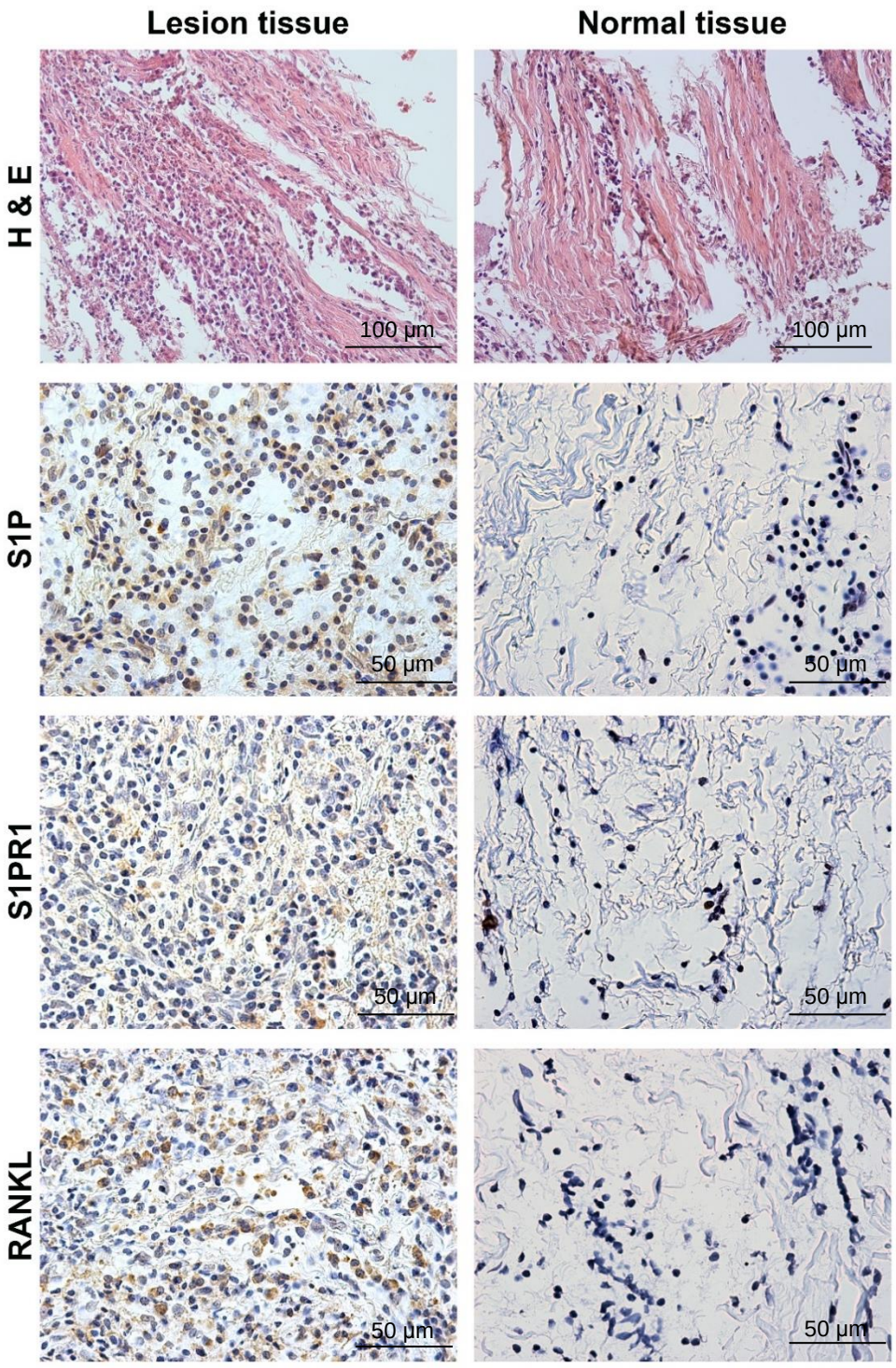


Figure 5. Histological and immunohistochemical observation of human lesion/normal periapical tissues (n=10). Representative views of histological (original magnification, 200×, Scale bar = 100 μm) and immunohistochemical observations (original magnification, 400×, Scale bar = 50 μm) in human periapical tissues. Compared with normal tissue, significantly enhanced immune cell infiltration could be observed in the lesions. The expression of S1P, S1PR1 was found to be up-regulated in periapical lesion tissues, suggesting the S1P-S1PR1 signalling was abnormally activated in apical periodontitis. Increased RANKL expression was observed in the lesion tissues, which could result in bone loss.

3.4.2 Identification of S1PR1$^+$ and RANKL$^+$ cells in human periapical lesions

To investigate the relation between S1P-S1PR1 signalling and RANKL expression, double-dye immunofluorescent staining of S1PR1 and RANKL was performed on the same section to detect the S1PR1$^+$ and RANKL$^+$ cells in periapical lesions. S1PR1$^+$ cells, RANKL$^+$ cells, as well as S1PR1$^+$RANKL$^+$ cells could be seen co-localizing in the periapical lesion tissues (arrows Fig 6). This suggested that the up-regulated S1P-S1PR1 signalling was associated with enhanced RANKL expression in periapical lesion tissues.

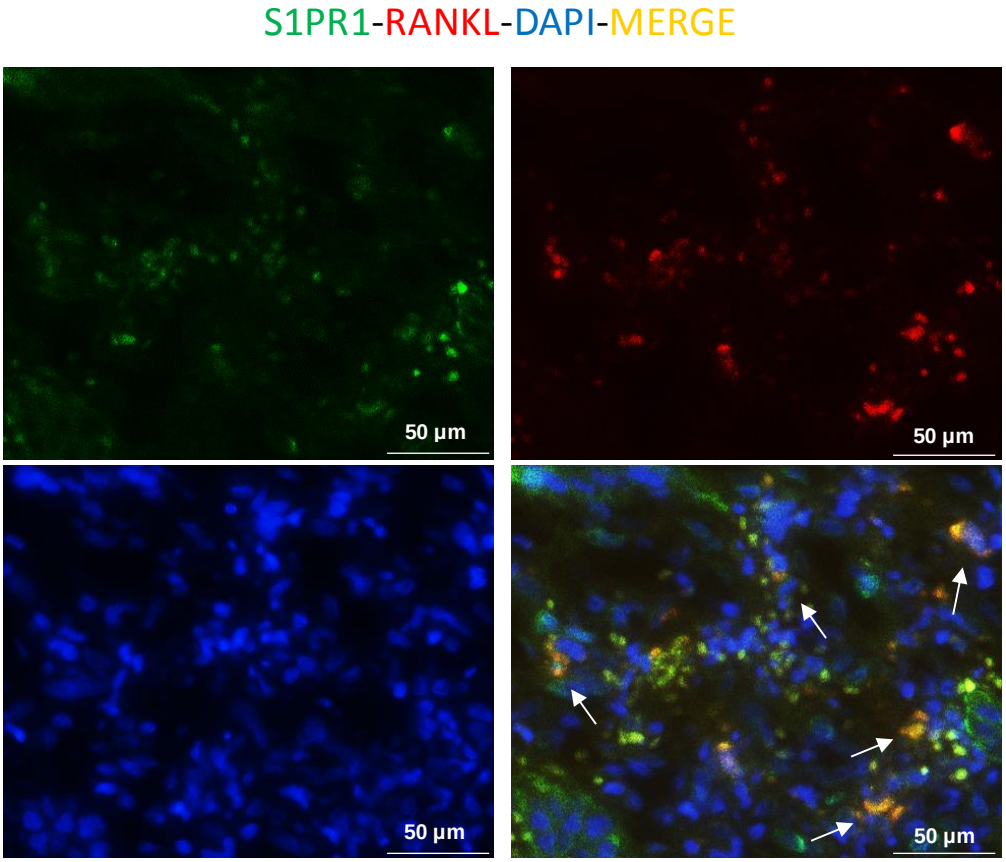


Figure 6. Immunofluorescent microscopy shows colocalization of S1PR1 and RANKL on human periapical lesions (n=10). S1PR1 was stained with Dylight488 (green), and RANKL was stained with CY3 (red). Nuclei were counterstained with DAPI (blue) (Original magnification, 200×, Scale bar = 50 μm). S1PR1$^+$RANKL$^+$ cells (yellow) could be seen in the periapical lesion tissues, suggesting the S1P-S1PR1 signalling was possibly related with RANKL expression in periapical lesions. Arrows indicate the S1PR1$^+$RANKL$^+$ cells.

3.4.3 The size of rat periapical lesion at different stages

To further study the involvement of S1P-S1PR1 signalling in the pathogenesis of apical periodontitis, animal model of this disease was induced on rats by pulp exposure (Stashenko, et al., 1994). The lesion area and volume were measured by high-resolution X-ray imaging and μCT. As shown in Fig 7, both the measurements of High-resolution X-ray imaging and μCT continued to increase after day 0 (Fig 7 b&d, Table 1), indicating the periapical area and volume expanded from day 0 to day 35. The periapical area (0.18 mm^2) and volume (0.36 mm^3) of group day 0 represented the normal periapical space of the first molar. According to clinical X-ray diagnose criteria, no lesions could be observed at this stage. From day 7 to day 21, the periapical lesion could be observed as the light blue area which grew in size (mean area grew from 0.34 mm^2 to 0.56 mm^2). Accordingly, the μCT results showed that the lesions formed and continuously broadened in the sagittal, horizontal, and coronal directions from day 7 to day 21 (mean volume grew from 0.73 mm^3 to 1.12 mm^3). From the measurements (Fig 7d), it could be found that the lesions continued to expand from day 28 to day 35, however, no significant differences of lesion volumes could be found on days 21, 28 and 35, indicated that the bone loss became comparably stable after day 21. A significant positive correlation was found between the area measured by high-resolution X-ray imaging and the volume by μCT ($r = 0.9872$, $p<0.01$). Data are shown in Table 1.

As shown in the histological slides (Fig 7a), pulp necrosis, infiltrating inflammatory cells, as well as alveolar bone resorption around the first molar apex gradually became more prominent over time following exposure of the dental pulp. The inflammatory cell infiltration was moderate on day 7, which became evident during day 14 to day 21, then turned to be less severe from day 28 to day 35. Same as the result of X-ray imaging (Fig 7c), the periapical area started to grow after day 0 (0.17 mm^2), which rapidly increased from day 7 (0.26 mm^2) to day 21 (0.59 mm^2) and became comparably stable during day 28 to day 35, as there were no significant differences between days 28 and 35.

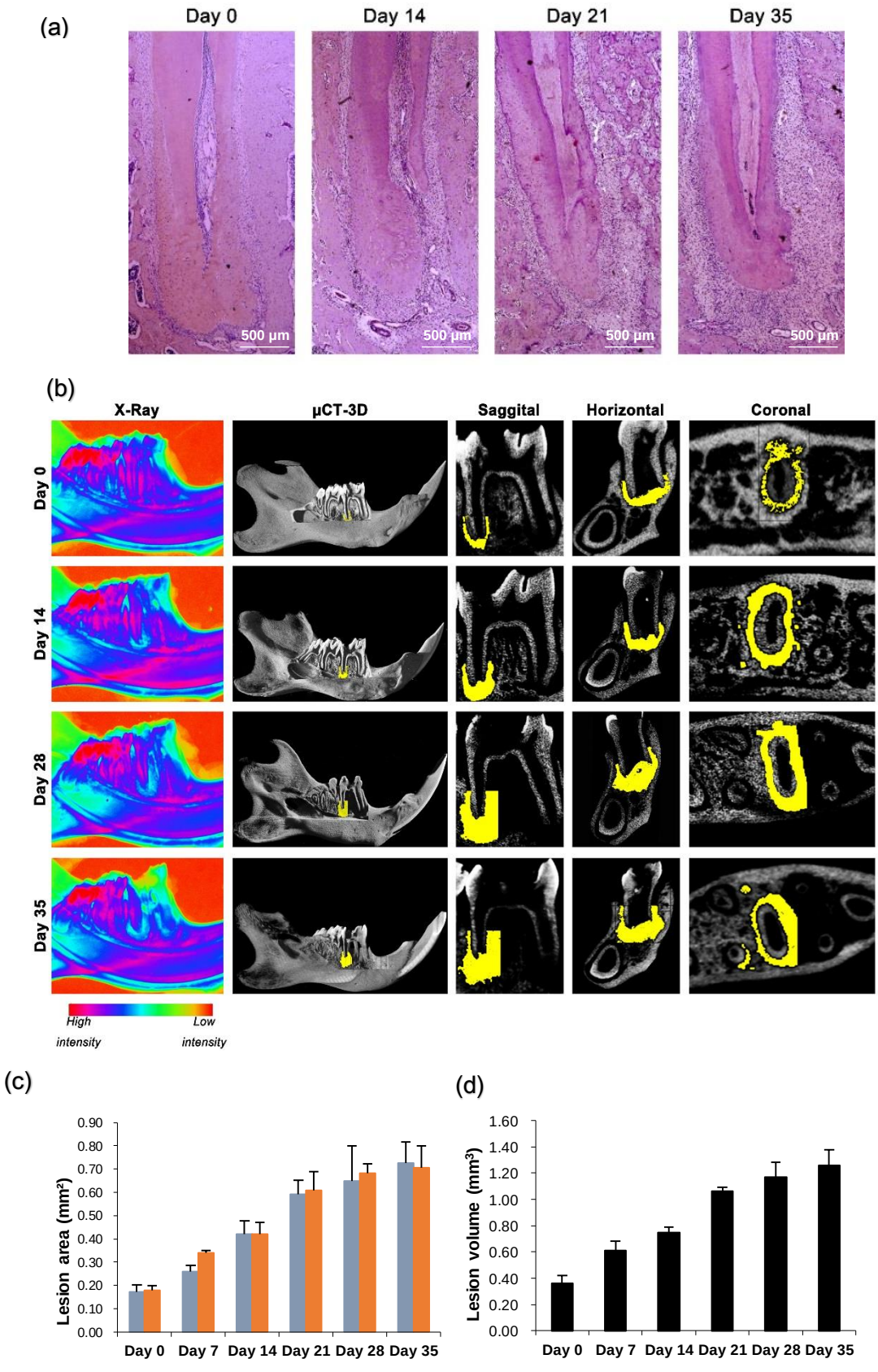

(a)
Day 0
Day 14
Day 21
Day 35
500 µm
(b)
X-Ray
µCT-3D
Saggital
Horizontal
Coronal
Day 0
Day 14
Day 28
Day 35
High intensity
Low intensity
(c)
Lesion area (mm²)
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Day 0
Day 7
Day 14
Day 21
Day 28
Day 35
(d)
Lesion volume (mm³)
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Day 0
Day 7
Day 14
Day 21
Day 28
Day 35

Figure 7. Identification of rat periapical lesion size on days 0, 7, 14, 21, 28, and 35 (n=5). (a) Histological observation of rat periapical lesions. Representative views of histological (original magnification, 50×, Scale bar = 500 μm) observation in rat periapical lesions on days 0, 14, 21, and 35. From days 7 to 35, the lesion area continuously increased, accompanied by inflammatory cell infiltration. (b) Periapical lesion size was analysed by high-resolution X-ray imaging and μCT. Different colours represented different levels of bone intensity. The intensity of the light blue area around the apex was comparably low. The entire mandible with the periapical lesions (marked yellow) on the first molar is shown in 3D. The first molar with the periapical lesions is shown in the sagittal, horizontal, and coronal directions. (c) Measurements of lesion size (mm^2) in X-ray imaging and histological analysis (n=5). The lesion area grew drastically from day 0 to day 21 and became comparably stable after day 28, as no significant difference ($p < 0.05$) could be found between days 28 and 35. (d) Measurements of periapical lesion volume (mm^3) (n=5). The lesion volume continuously grew from day 0 to day 2 and became stale thereafter, as no significant differences (p <0 .05) were found on day 21, day 28 and day 35.

Table 1. Bone resorption volumes and areas of rat periapical lesions (mean±standard deviation)

Group	N	Lesion area (mm^2)[I] (HE)	Lesion volume(mm^3)	Lesion area (mm^2)[II] （X-RAY ）
Day 0	5	$0.18 \pm 0.02^{*,\#,\dagger,£,‡}$	$0.36 \pm 0.06^{*,\#,\dagger,£,‡}$	$0.17 \pm 0.03^{*,\#,\dagger,£,‡}$
Day 7	5	$0.34 \pm 0.01^{\#,\dagger,£,‡,\&}$	$0.61 \pm 0.07^{\#,\dagger,£,‡,\&}$	$0.26 \pm 0.03^{\#,\dagger,£,‡,\&}$
Day 14	5	$0.40 \pm 0.05^{*,\dagger,£,‡,\&}$	$0.75 \pm 0.04^{*,\dagger,£,‡,\&}$	$0.42 \pm 0.06^{*,£,‡,\&}$
Day 21	5	$0.61 \pm 0.12^{*,\#,‡,\&}$	$1.06 \pm 0.03^{*,\#,\&}$	$0.59 \pm 0.06^{*,£,‡,\&}$
Day 28	5	$0.68 \pm 0.04^{*,\#,\&}$	$1.17 \pm 0.11^{*,\#,\&}$	$0.65 \pm 0.15^{*,\#,\&}$
Day 35	5	$0.70 \pm 0.09^{*,\#,\dagger,\&}$	$1.26 \pm 0.12^{*,\#,\&}$	$0.73 \pm 0.09^{*,\#,\dagger,\&}$

* $p < 0.05$ versus 7-day group. # $p < 0.05$ versus 14-day group. † $p < 0.05$ versus 21-day group. £ $p < 0.05$ versus 28-day group. ‡ $p < 0.05$ versus 35-day group. & $p < 0.05$ versus 0-day group (control). I Positive correlation (r = 0.9872, $p < 0.01$) with lesion volume. II Positive correlation (r = 0.9852, $p < 0.01$) with Lesion area (X-RAY).

3.4.4 Up-regulated S1PR1 expression in rat periapical lesions

To investigate the expression of S1PR1in the pathogenesis of apical periodontitis, IHC staining was performed at different time points of rat periapical lesions. As shown in Fig 8, there were few if any $S1PR1^+$ cells (except for endothelial cells) in the day 0 periapical tissues, whereas S1PR1 expression became evident from day 7 to day 35. Similar to what was seen in the human tissues, a large quantity of $S1PR1^+$ cells appeared to be lymphocytes, macrophages and leukocytes, accompanied by the fibroblast-like cells. The $S1PR1^+$ endothelial cells could also be observed.

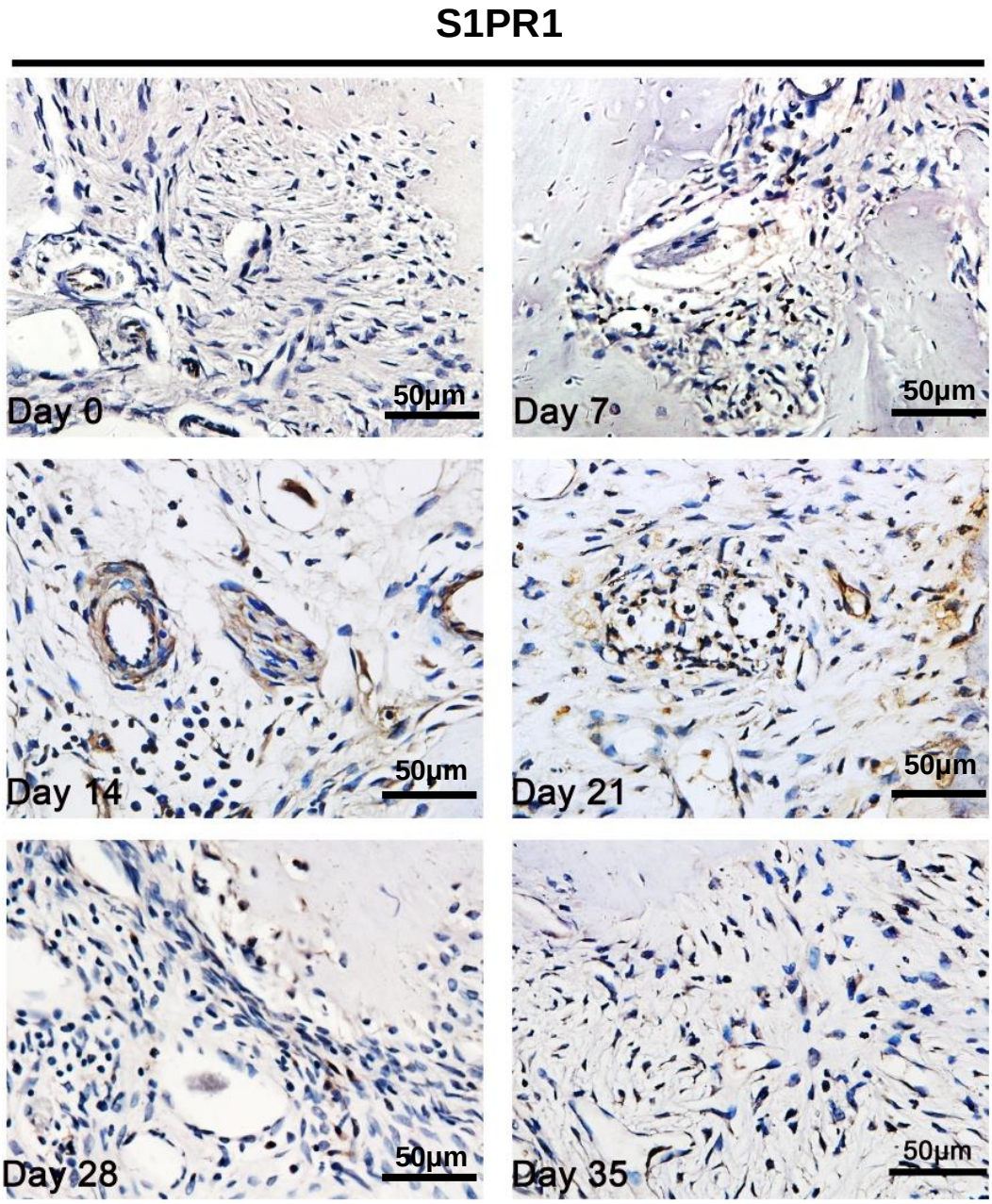


Figure 8. Immunohistochemical observation of S1PR1-expression in rat periapical lesions (original magnification, 400×, Scale bar = 50 μm, n=5). No S1PR1-positive cells were found on day 0. The expression of S1PR1 increased from days 7 to 14, peaked from day 14 to day 21, then decreased from days 28 to 35.

3.4.5 S1PR1, RANKL, and osteoclast expression dynamics in rat periapical lesions

To detect the relation between S1PR1 and RANKL in the pathogenesis of apical periodontitis, IHC staining against RANKL was performed, and the expression of RANKL was compared with that of S1PR1. Compared to day 0, the RANKL expression was significantly enhanced during day 7 to day 35 (Fig 9). Cell counting showed the numbers of $S1PR1^+$ cells and $RANKL^+$ cells to be low at day 7, which dramatically increase from day 7 to day 14 and remain at a high level from day 14 to day 21, after which there was a decreased number of positive cells that persisted after day 28 (Fig 11, Table 2). The cell counts and the corresponding standard deviation values from days 0 to 35 are listed in Table 2.

TRAP staining was performed to detect the expression of osteoclasts during the pathogenesis of apical periodontitis. On day 0, no osteoclasts could be observed, while the expression of osteoclasts became evident from day 7 to day 35 (Fig 10). According to the cell counting results, the number of osteoclasts was low at day 7 (Fig 11), then peaked on day 14, and decreased from day 21 to day 35. Data are shown in Table 2.

To find the relation of S1PR1 with RANKL and osteoclastogenesis in apical periodontitis, we compared the expression of S1PR1, RANKL and osteoclasts. As shown in Fig 11, the dynamic expression of S1PR1 was similar to that of RANKL and osteoclasts. From day 0 to day 35, a significant positive correlation was found between S1PR1 and RANKL-positive cells ($r = 0.9171$, $p < 0.01$, Fig 11). The same correlation was found between $S1PR1^+$ cells and TRAP-positive cells ($r = 0.9535$, $p < 0.01$). Data are shown in Table 2.

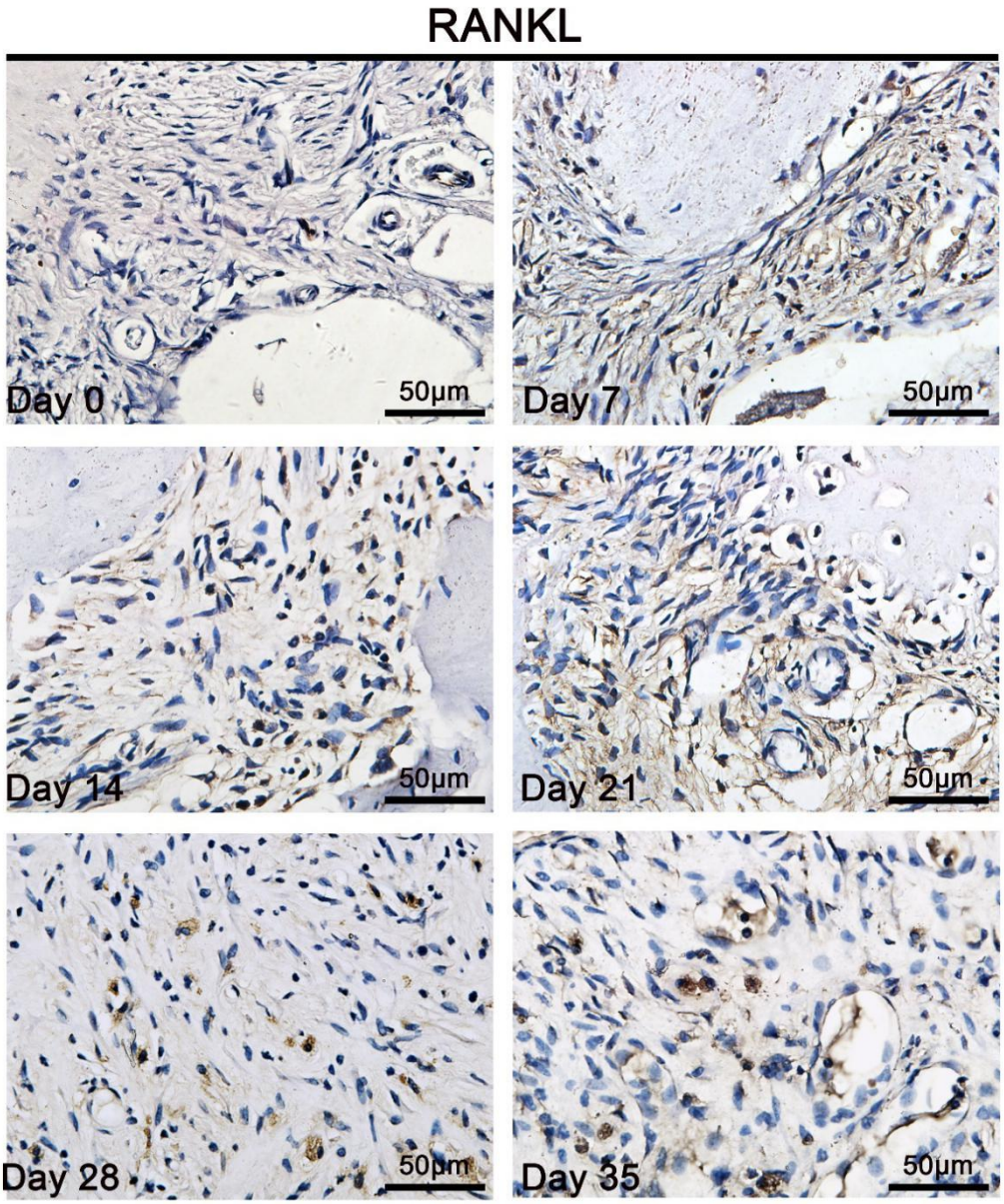


Figure 9. Immunohistochemical observation of RANKL-expression in rat periapical lesions (original magnification, 400×, Scale bar = 50 μm, n=5). No RANKL-positive cells were found on day 0. The RANKL expression was low on day 7, then increased from day 7 to day 14, peaked from day 14 to day 21, decreased and became comparably stable after day 28.

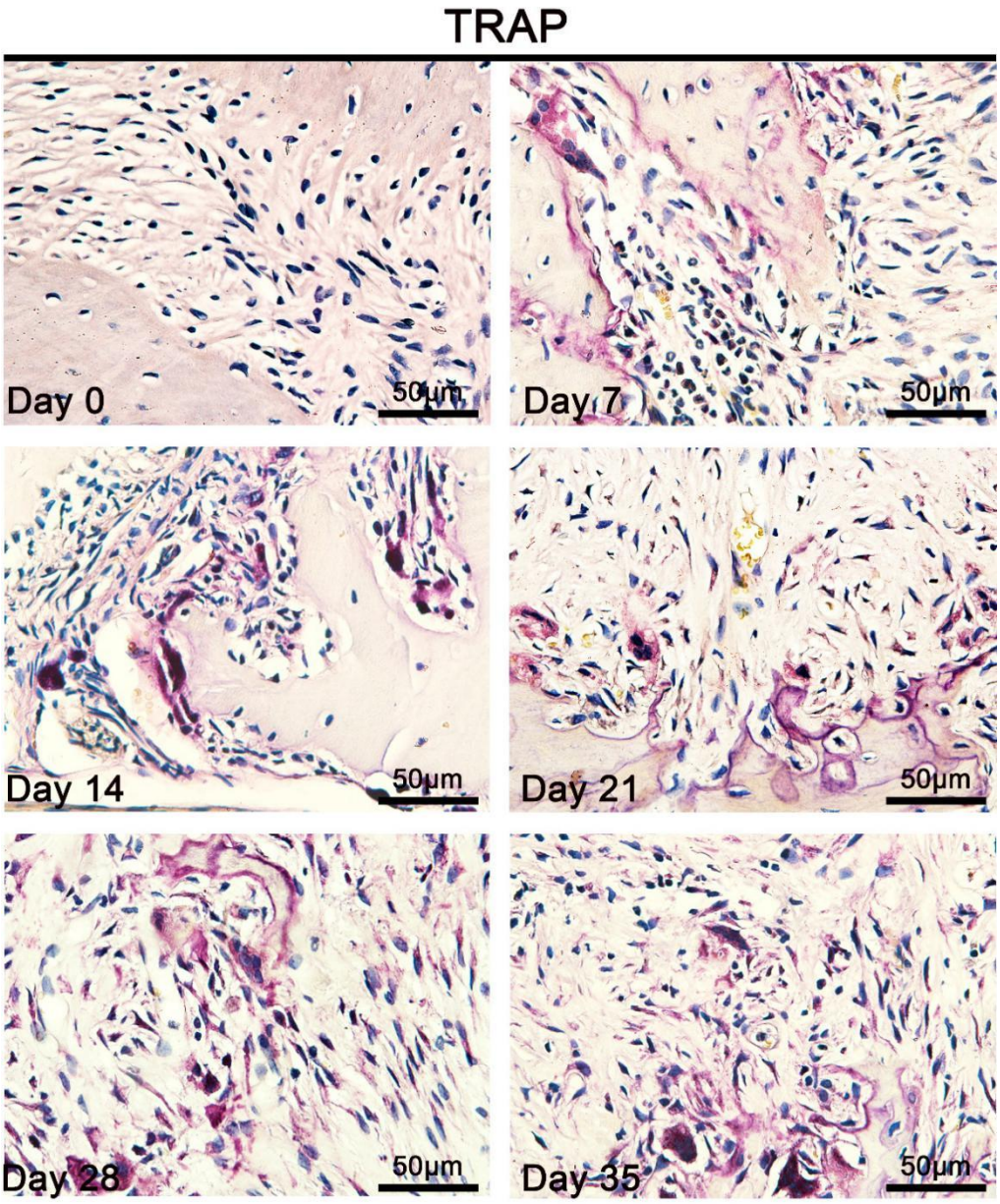


Figure 10. Enzyme histochemical observation of rat periapical lesions (original magnification, 400×, Scale bar = 50 μm, n=5). No TRAP-positive cells were found on day 0. TRAP-positive cells appeared on day 7; the expression of TRAP-positive cells peaked from day 14 to day 21 and decreased thereafter.

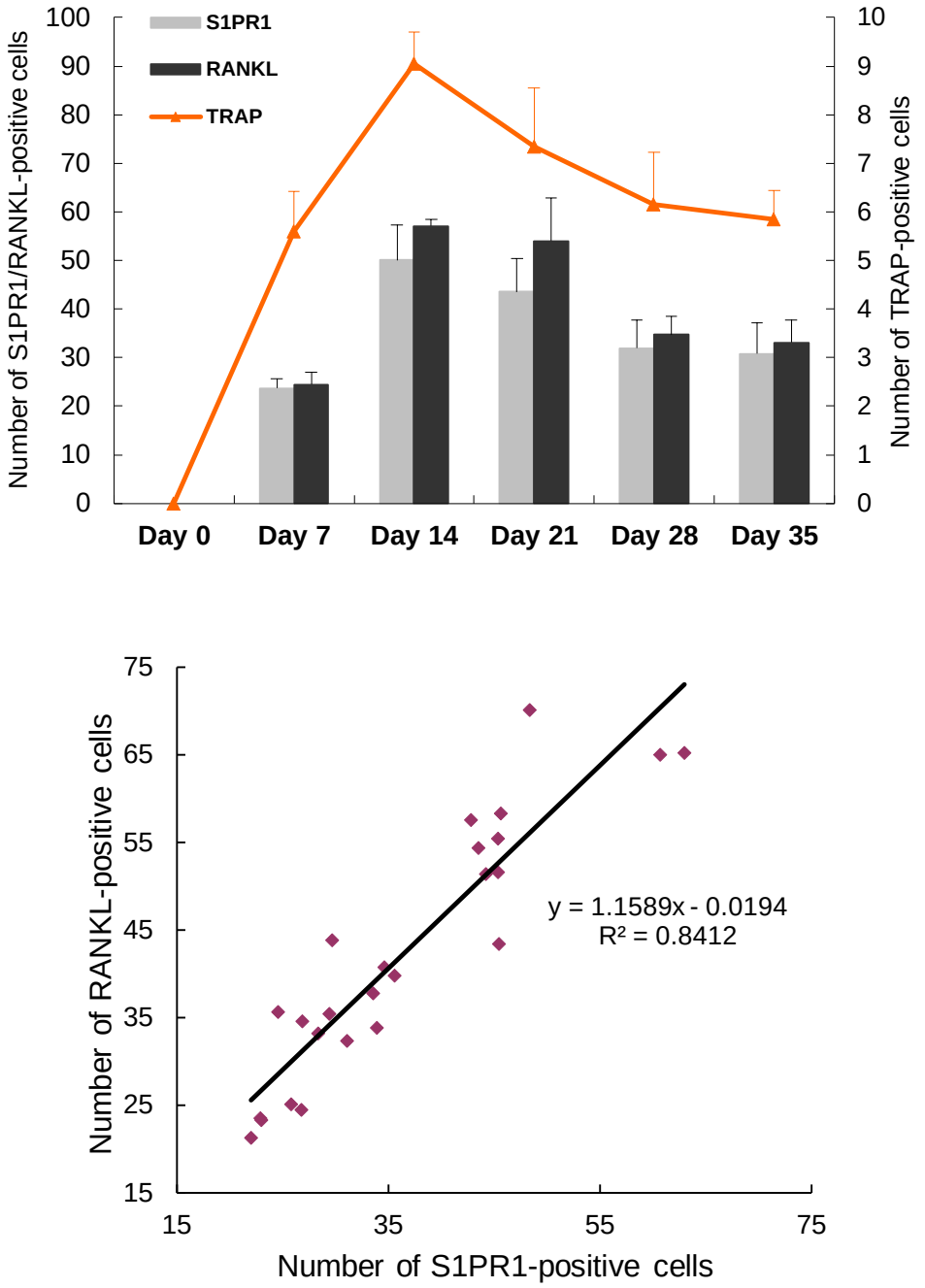


Figure 11. Measurements of TRAP/S1PR1/RANKL positive cells in rat periapical lesions (n=5). The number of osteoclasts increased from days 0 to 14 and then decreased from days 21 to 35. The numbers of S1PR1- and RANKL-positive cells increased from days 0 to 14 and then decreased from days 21 to 35. Significant differences were observed between each time point ($p< 0.05$), except between days 14 and 21 and between days 28 and 35. Positive correlation existed between the expression of S1PR1 and RANKL in rat periapical lesions (r = 0.9171, $p<0$ .01).

Table 2. Numbers of S1PR1$^+$ cells, RANKL$^+$ cells and osteoclasts per high-power field (hpf; 400×).

Group	N	S1PR1$^+$ cells/hpf	RANKL$^+$ cells/hpf[I]	Osteoclasts/hpf[II]
Day 0	5	—	—	—
Day 7	5	23.70 ±1.95$^{\#,\dagger,£,‡}$	24.43 ± 2.52$^{\#,\dagger,£‡,}$	5.60 ± 0.83$^{\#,\dagger,£,‡}$
Day 14	5	50.03 ± 7.27$^{*,£,‡}$	57.03 ± 1.43$^{*,£,‡}$	9.05 ± 0.65$^{*,£,‡}$
Day 21	5	43.56 ± 6.82$^{*,£,‡}$	53.95 ± 8.89$^{*,£,‡}$	7.34 ± 1.20$^{*,£,‡}$
Day 28	5	31.88 ± 5.87$^{*,\#,\dagger}$	34.74 ± 3.80$^{*,\#,\dagger}$	6.15 ± 1.08$^{*,\#,\dagger}$
Day 35	5	30.76 ± 6.43$^{*,\#,\dagger}$	33.06 ± 4.71$^{*,\#,\dagger}$	5.84 ± 0.60$^{*,\#,\dagger}$

* p<0.05 versus 7-day group. # p <0.05 versus 14-day group. † p <0.05 versus 21-day group. £ p <0.05 versus 28-day group. ‡ p < 0.05 versus 35-day group. & p <0.05 versus 0-day group (control). I Positive correlation (r = 0.9171, p < 0.01) with numbers of S1P1$^+$ cells/hpf. II Positive correlation (r =0.9535, p < 0.01) with numbers of S1P1$^+$ cells/hpf.

3.4.6 Immunofluorescent co-localization of S1PR1 and RANKL in rat periapical lesions

To confirm the relation between S1PR1 and RANKL, double-dye immunofluorescent staining of S1PR1 and RANKL was performed in different time points of rat periapical lesions. As shown in Fig 12, a number of S1PR1$^+$ (green) cells and RANKL$^+$ (red) cells could be observed in rat periapical lesions. Similar to what was seen in the human tissues, a number of S1PR1$^+$ cells overlapped with RANKL$^+$ cells in the periapical region on days 14, 21, and 35 in the rat periapical lesions. This further suggests that S1PR1 activation is related with RANKL expression in the pathogenesis of apical periodontitis.

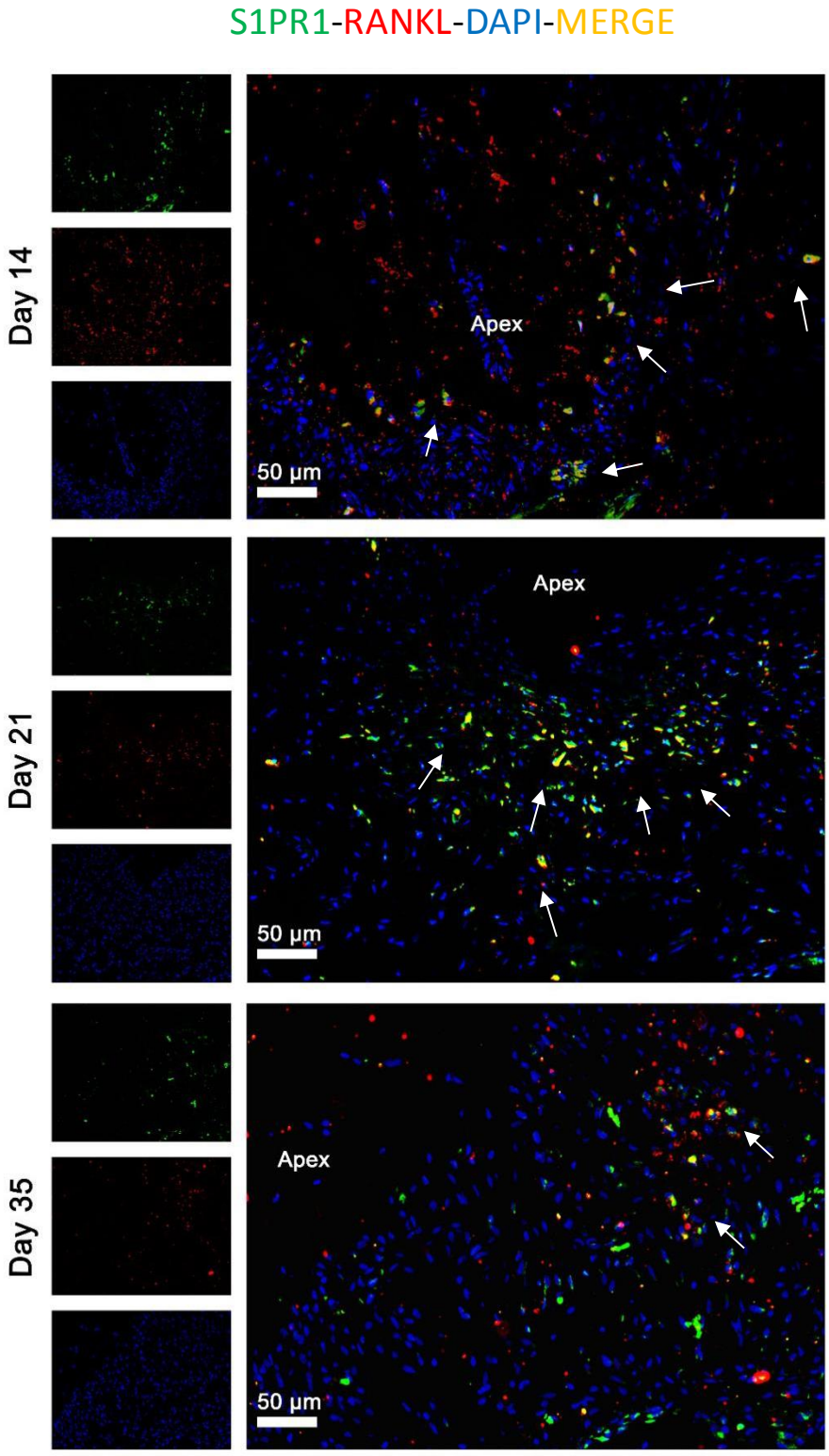


Figure 12. Immunofluorescent colocalization of S1PR1 and RANKL on days 14, 21, and 35 (n=5). S1PR1 was stained with Dylight488 (green), and RANKL was stained with CY3 (red). Nuclei were counterstained with DAPI (blue). (Original magnification, 200×, Scale bar = 50 μm). $S1PR1^{+}RANKL^{+}$ cells (yellow) could be observed in different time points (days 14, 21, and 35) of rat periapical lesions, suggesting S1PR1 activation was related with RANKL expression in the pathogenesis of apical periodontitis. Arrows indicate the $S1PR1^{+}RANKL^{+}$ cells.

3.4.7 S1P-S1PR1 signalling modulation limited periapical lesions exacerbation

The role of S1P-S1PR1 signalling in apical periodontitis was further studied by its modulation (administration of FTY720). Histological analysis (H&E staining) was used for general morphology and measurements of bone loss (lesion area); sections were observed under both fluorescence and light microscopes. As shown in Fig 12, periapical bone destruction and lesion formation appeared in both of the control and experimental groups, accompanied with pulp necrosis and inflammatory cell infiltration. Apart from the control group, of which lesion areas greatly grew up from day 7 to day 35, the lesion areas of experimental group were comparably stable and much smaller. As shown in Fig 12 and Table 3, since day 7, the lesions of control group were significantly larger than experimental group. All the data above indicated that the pathogenesis of periapical lesions was effectively reduced by S1P-S1PR1 signalling intervention.

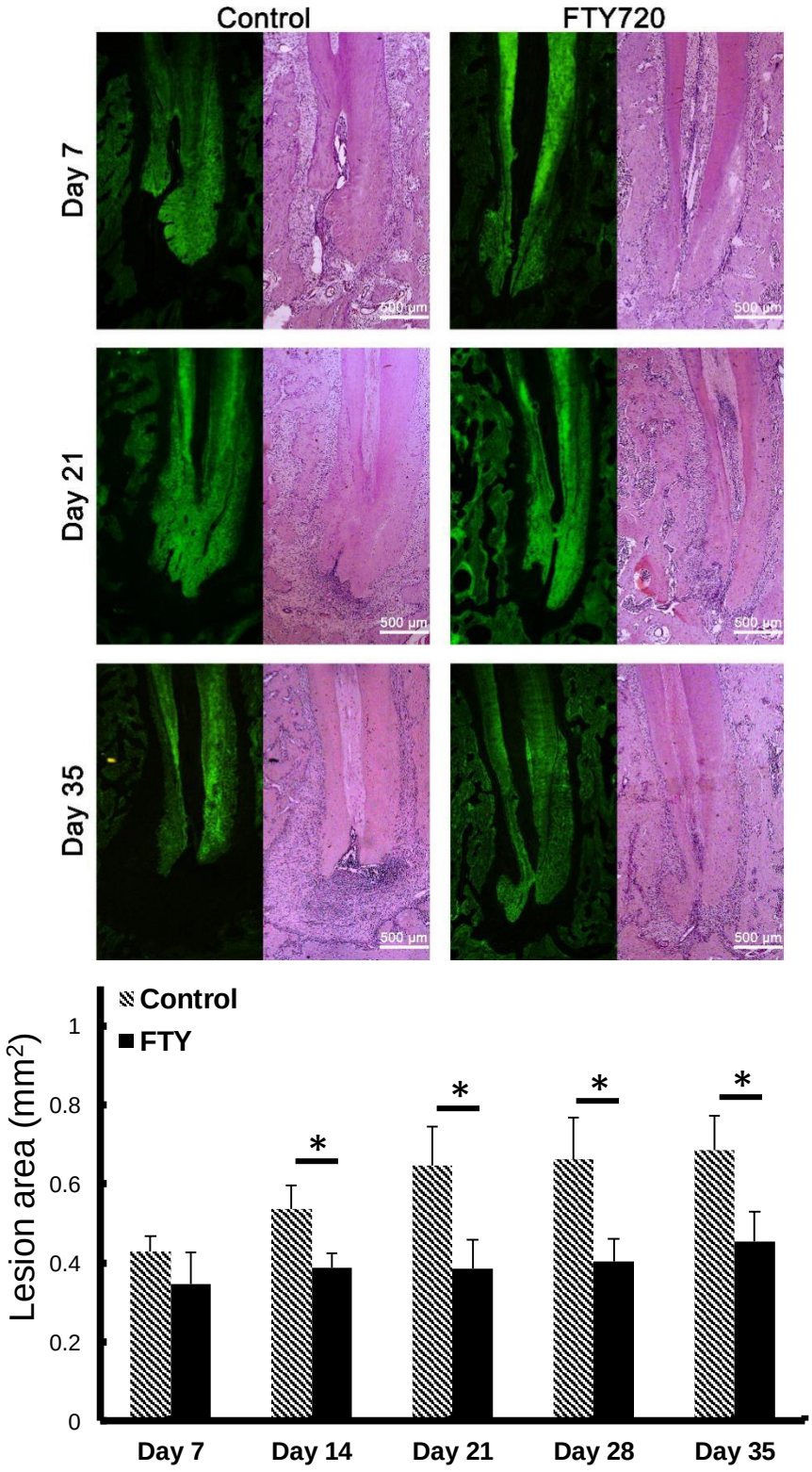
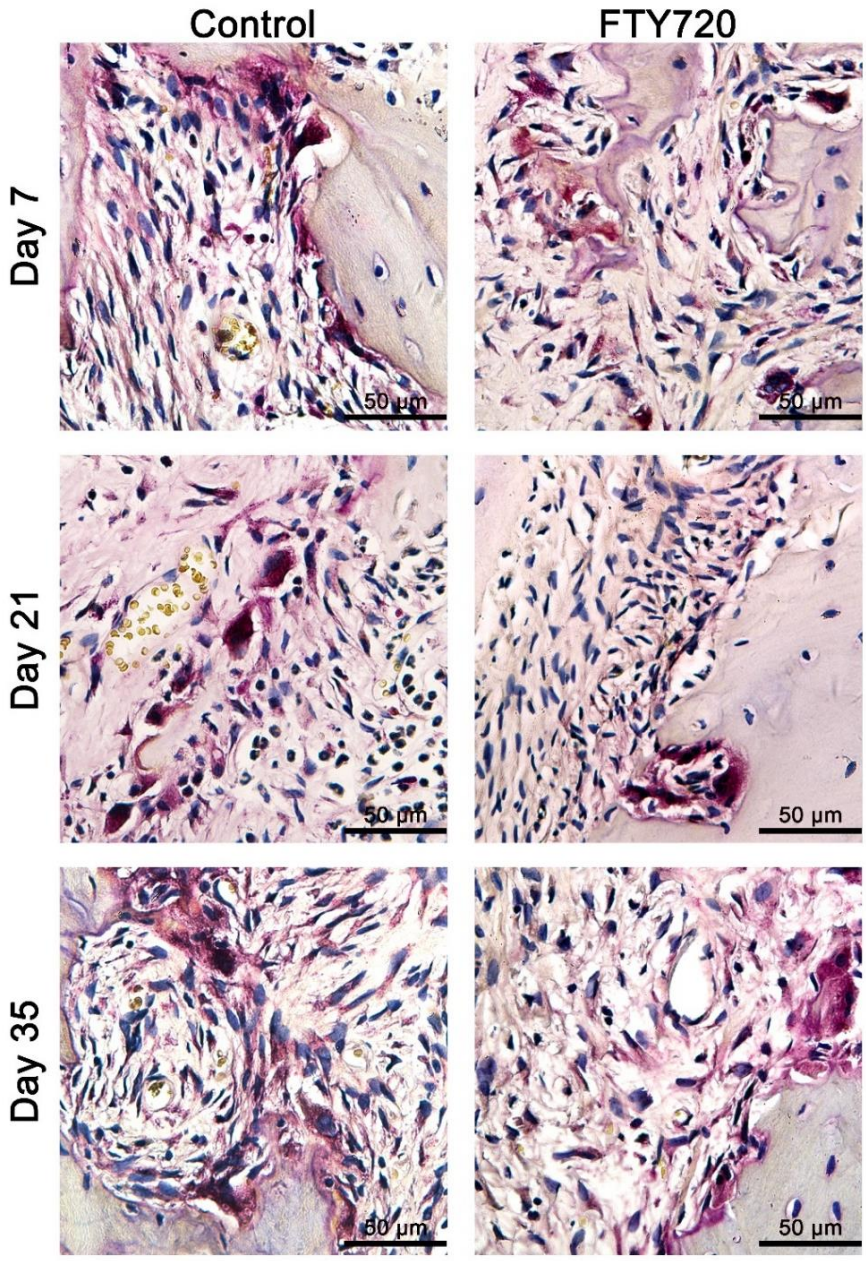


Figure 13. S1P-S1PR1 signalling reduced bone resorption in apical periodontitis (n=5). Periapical lesion size was analysed by histological staining (observed under both fluorescence and light microscopes) on days 7, 21, and 35 (original magnification, 50×, Scale bar = 500μm). In the control group, the lesion area greatly grew up from day 7 to day 35; while the lesion area of FTY720 administration group was comparably stable and much smaller than that of control group; the measurements indicated that the lesion area of control group was larger than that of FTY720 group at each time point, especially after day 7. Significant differences were observed between two groups at each time point except for day 7 (*p <0.05). All the data above indicated that the bone loss in apical periodontitis was effectively reduced by intervening S1P-S1PR1 signalling.

3.4.8 S1P-S1PR1 signalling modulation reduced osteoclastogenesis in apical periodontitis

The TRAP staining was performed to investigate the impacts of S1P-S1PR1 signalling modulation on osteoclastogenesis. As shown in Fig 14 and Table 3, from day 7 to day 35, the osteoclast expression of experimental group was significantly lower than that of control group. FTY720 administration effectively restrained osteoclast expression and kept it on a comparably low level; while in the control group the osteoclast expression peaked on day 14 and then gradually decreased.



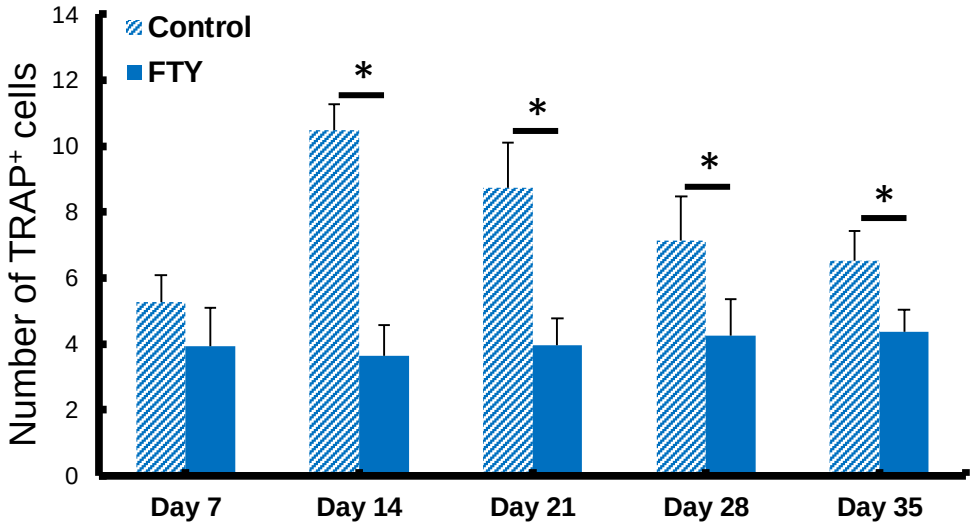


Figure 14. S1P-S1PR1 signalling modulation reduced osteoclastogenesis in apical periodontitis (n=5). It could be observed that from day 7 to day 35, the expression of osteoclasts in control group was higher than that in FTY720 group; which was further confirmed by cell counting results: the osteoclast numbers of control group was higher than that of FTY720 group at each time point (original magnification, 400×, Scale bar = 50 μm); significant differences were observed between two groups at each time point except for day 7 (*p<0.05). All the data above indicated that the osteoclastogenesis in apical periodontitis was effectively reduced by intervention of S1P-S1PR1 signalling.

3.4.9 S1P-S1PR1 signalling modulation down-regulated RANKL expression, while caused no effect on OPG expression

IHC staining was performed to investigate the impacts of S1P-S1PR1 signalling modulation on RANKL and OPG expression. After day 7, the S1PR1 and RANKL expression was significantly reduced following FTY720 administration (as shown in Fig 15, 16 and 18). The cell counting results showed that both S1PR1 and RANKL expression levels remained low from day 7 to day 21 and increased only slightly from day 28 to day 35 (Fig 18). This was in contrast to the control group, in which S1PR1 expression along with that of RANKL reached peak expression on day 14 and decreased thereafter (Fig 18). The OPG expression (Fig 17), however, was almost the same between two groups, as there was no significant difference between the control and experimental groups (Fig 18). The ratio of RANKL/OPG positive cells was calculated; as shown in Fig 18, the ratio of RANKL/OPG was decreased after FTY720 administration, indicated that the inhibition of S1P-S1PR1 signalling reduced the RANKL expression, therefore attenuated the imbalance of RANKL and OPG in the development of apical periodontitis.

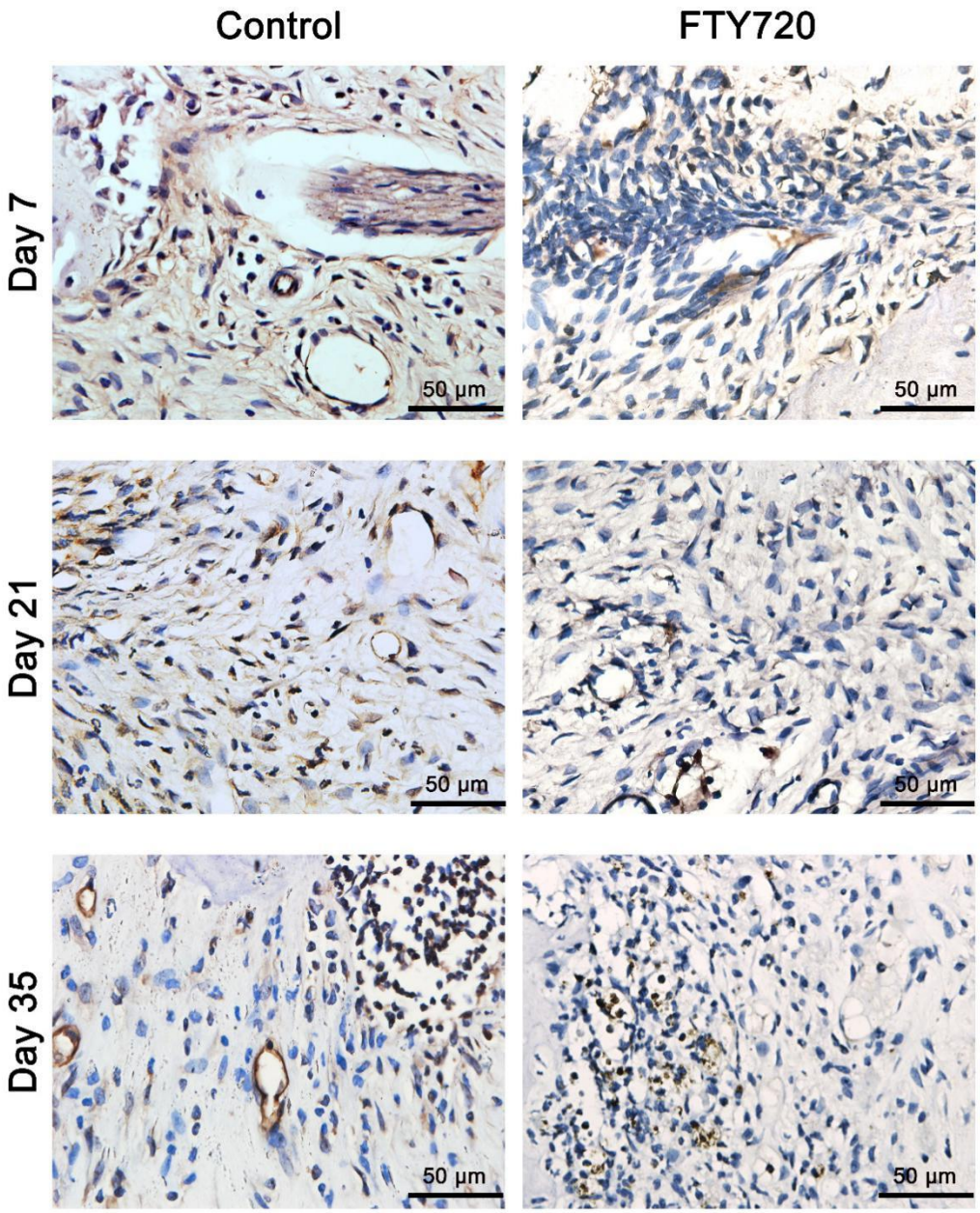


Figure 15. FTY720 down-regulated S1PR1 expression in apical periodontitis (n=5). From the IHC staining results, it could be observed that the expression of S1PR1 in control group was higher than that in FTY720 group at each time point (original magnification, 400×, Scale bar = 50 μm), indicated the S1P-S1PR1 signalling was successfully inhibited by FTY720 administration.

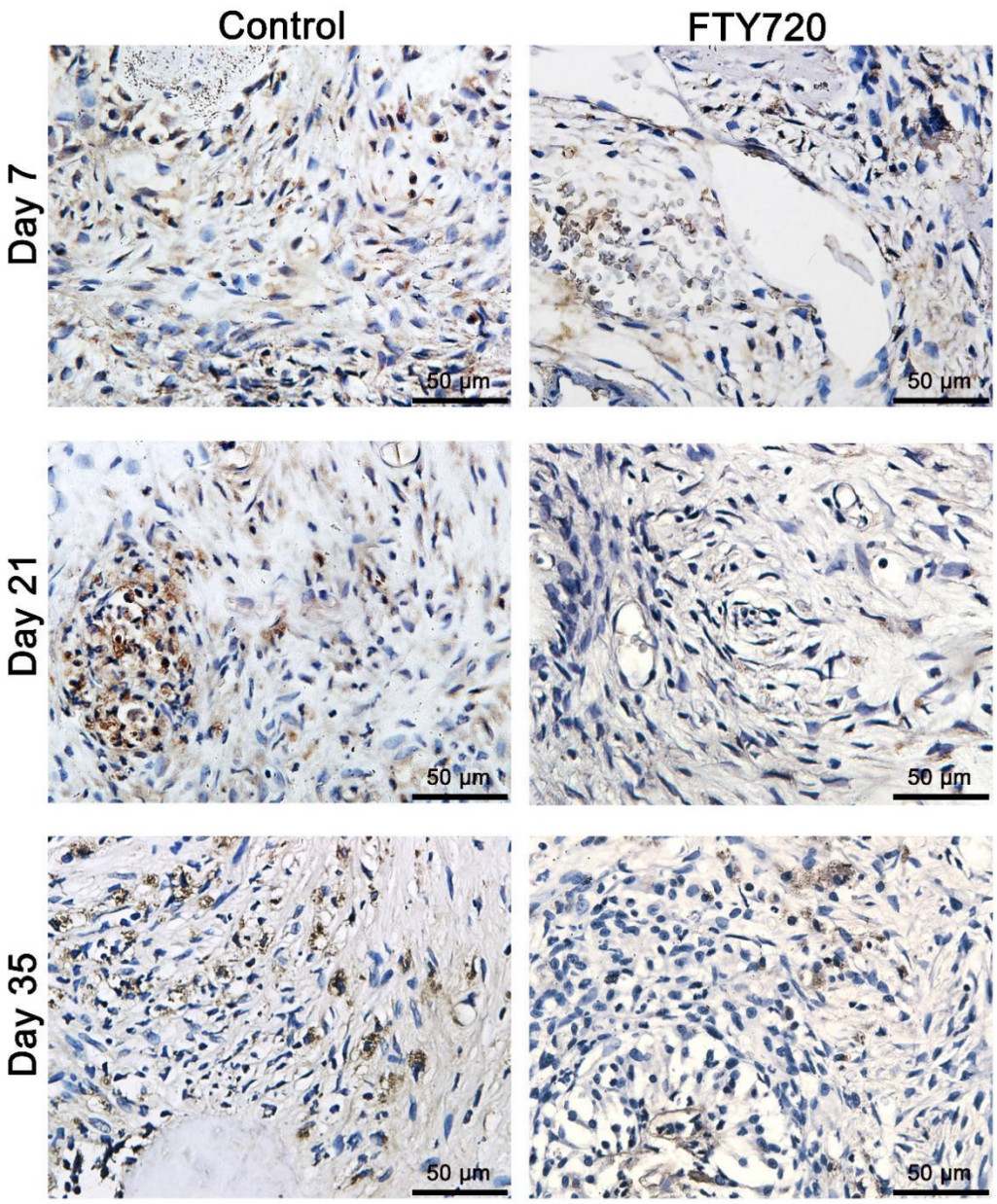


Figure 16. FTY720 reduced RANKL expression in apical periodontitis (n=5). From the IHC staining results, it could be observed that the RANKL expression of control group was higher than that of FTY720 group at each time point (original magnification, 400×, Scale bar = 50 μm), indicated that inhibiting S1P-S1PR1 signalling leaded to decreased RANKL expression in the pathogenesis of apical periodontitis.

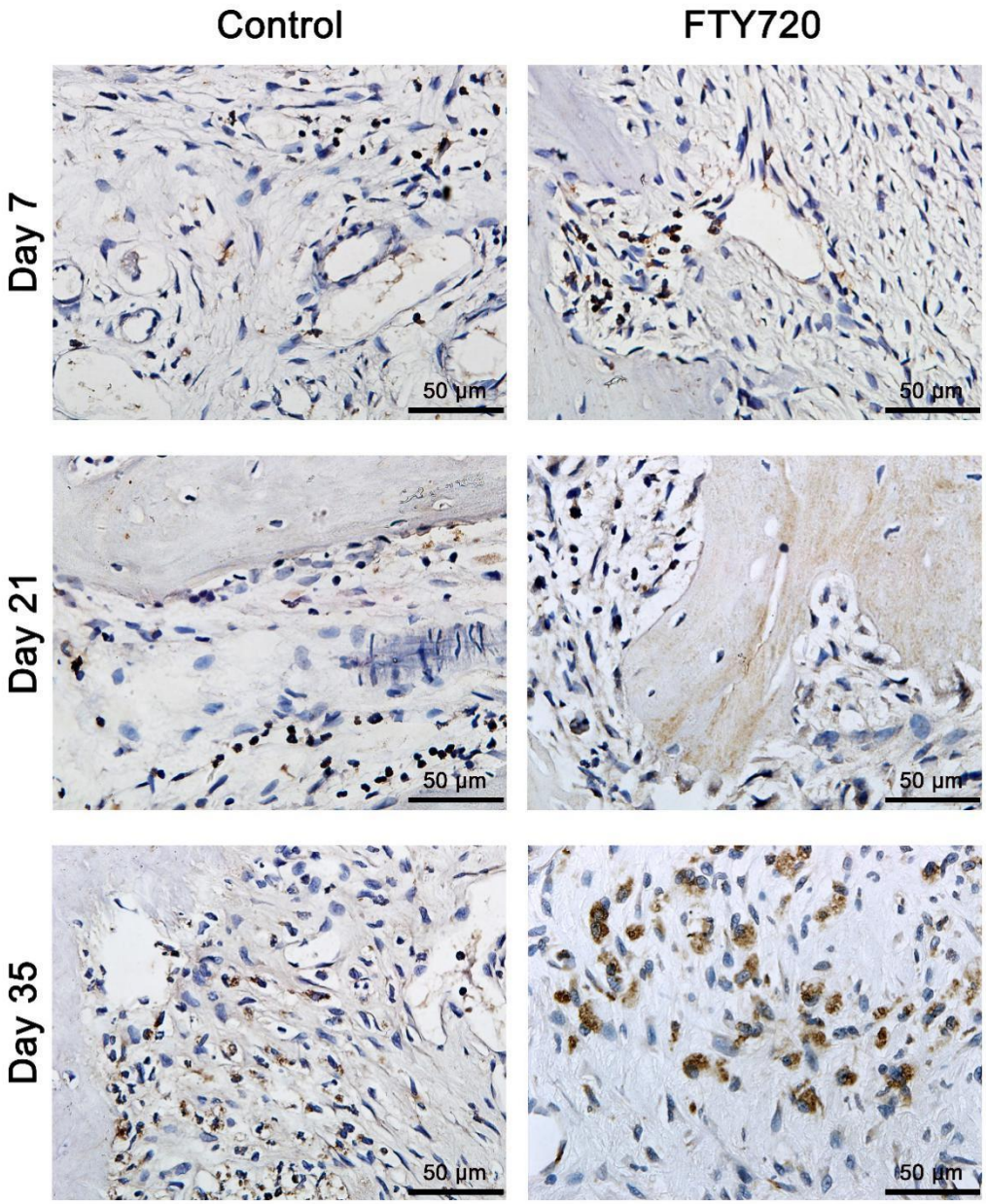


Figure 17. FTY720 caused no effect on OPG expression in apical periodontitis (n=5). From the IHC staining results, no difference on OPG expression could be observed between the control group and FTY720 group at each time point (original magnification, 400×, Scale bar = 50 μm), indicated that the inhibition of S1P-S1PR1 signalling would not affect the OPG expression in the development of apical periodontitis.

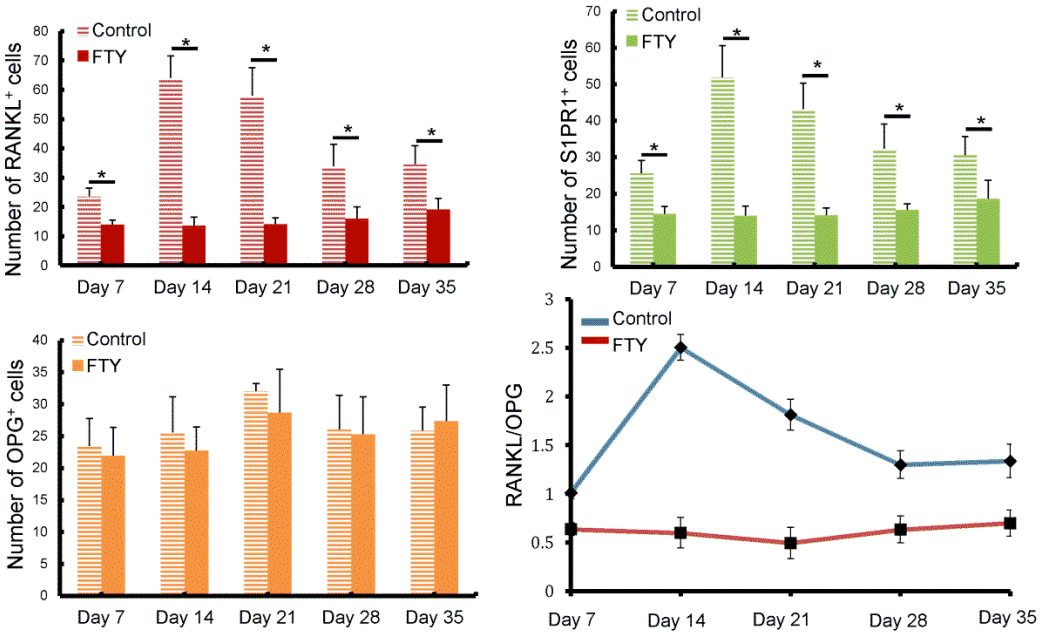


Figure 18. Measurements of S1PR1/RANKL/OPG positive cells in rat periapical lesions (n=5). The numbers of S1PR1- and RANKL-positive cells in control group were much higher than that in FTY720 group at each time point, suggested that inhibited S1PR1 resulted in reduced RANKL expression in apical periodontitis. Significant differences were observed between two groups at each time point (*p< 0.05). For OPG-positive cells, no significant differences were found between two groups at each time point (p>0.05). The ratio of RANKL/OPG in control group was much higher than that in FTY720 group, indicated that the intervention of S1P-S1PR1 signalling attenuated the imbalanced RANKL/OPG in the pathogenesis of apical periodontitis.

Table 3. Numbers of S1PR1$^+$ cells, RANKL$^+$ cells, and osteoclasts per high-power field (hpf; 400×), areas of periapical lesions (mean ± standard deviation)

Group	N	S1PR1$^+$ cells/hpf	RANKL$^+$ cells/hpf	Osteoclasts/hpf	Lesion area (mm^2)
Day 7 (control)	5	25.65±3.44$^{\#,\dagger,£,‡}$	23.70 ± 2.74$^{\#,\dagger,£‡,}$	5.28 ± 0.81$^{\#,\dagger,£,‡}$	0.43 ± 0.04$^{\#,\dagger,£,‡}$
Day 7 (FTY)	5	14.50 ±1.99^{I}	13.96 ± 1.49^{I,‡}	3.93 ± 1.18	0.35 ± 0.08$^{£,‡}$
Day 14 (control)	5	51.80 ± 8.78$^{I,*,£,‡}$	64.05 ± 7.46$^{I,*,£,‡}$	10.50 ± 0.77$^{I,*,£,‡}$	0.53 ± 0.06$^{I,*,\dagger,£,‡}$
Day 14 (FTY)	5	14.01 ± 2.64^{I}	13.62 ± 2.90^{I,‡}	3.65 ± 0.93$^{\#}$	0.39 ± 0.04^{I}
Day 21 (control)	5	43.12 ± 7.16$^{*,£,‡}$	58.03 ± 9.55$^{*,\#,\dagger}$	8.75 ± 1.37$^{*,£,‡}$	0.65± 0.09$^{*,\#}$
Day 21 (FTY)	5	14.10 ± 2.04^{I}	14.16 ± 2.05^{I,‡}	3.95 ± 0.82^{I}	0.38± 0.07^{I}
Day 28 (control)	5	32.31 ± 6.82$^{*,\#,\dagger}$	33.87 ± 7.57$^{*,\#,\dagger}$	7.13 ± 1.35$^{*,\#,\dagger}$	0.66 ± 0.10$^{*,\#}$
Day 28 (FTY)	5	15.63 ± 1.63^{I}	15.99 ± 4.05^{I}	4.25 ± 1.11^{I}	0.39 ± 0.05$^{I,*,\#,\dagger}$
Day 35 (control)	5	30.51 ± 5.21$^{*,\#,\dagger}$	34.61 ± 6.27$^{*,\#,\dagger}$	6.53 ± 0.89$^{*,\#,\dagger}$	0.68 ± 0.08$^{*,\#}$
Day 35 (FTY)	5	18.68 ± 4.95^{I}	19.08 ± 3.78$^{I,*,\#,\dagger}$	4.36 ± 0.66^{I}	0.45 ± 0.06$^{I,*,\#,\dagger}$

I p< 0.05 versus control group. * p < 0.05 versus 7-day. # p < 0.05 versus 14-day. † p < 0.05 versus 21-day. £ p < 0.05 versus 28-day. ‡ p < 0.05 versus 35-day.

3.5 DISCUSSION

The pathogenesis of apical periodontitis, which consists of both periapical inflammation and alveolar bone destruction, is characterized by the infiltration of immune cells, such as lymphocytes, macrophages, leukocytes, plasma cells and mast cells (Marton & Kiss, 2000; Silva, Garlet, Fukada, Silva, & Cunha, 2007; Stashenko, et al., 1998). Among these cells, T cells are believed to play the pivotal role, which support osteoclastogenesis by producing RANKL (Kawashima, Okiji, Kosaka, & Suda, 1996; Matsuo, et al., 1992; Theill, et al., 2002), the key osteoclastogenic cytokine in osteoclast differentiation and periapical lesion formation (Yasuda, et al., 1999; Zhang & Peng, 2005). S1P, a bioactive metabolite of plasma‐membrane sphingolipids, is of great importance in immune response, which regulates immune cell migration, function, polarization and cytokine production by binding with a family of G-protein-coupled receptors (S1PR1-S1P5) (Maceyka, et al., 2012; Spiegel & Milstien, 2011). S1PR1, the main S1P receptor that facilitates the infiltration and retention of effector T cells in the inflammatory tissues, plays a central role in inflammatory response (Rivera, et al., 2008). The S1P-S1PR1 signalling also participates in modulating the differentiation, function, and migration of osteoclasts (Ishii, et al., 2009). Our present study investigated the expression of S1P-S1PR1 signalling in both human and rat periapical lesions and its role in apical periodontitis-derived bone loss.

In this study, firstly, the status of S1P-S1PR1 signalling was examined in human periapical lesion tissues. The immunohistochemical staining results showed that compared with the normal periapical tissues, both S1P and S1PR1 expressions were enhanced in the lesion tissues, indicating that S1P-S1PR1 signalling was induced during the pathological development of apical periodontitis. To find out whether this signalling took part in the pathogenesis of bone destruction, a double-dye immunofluorescent staining of S1PR1 and RANKL was performed to detect the relationship of S1P-S1PR1 signalling to the expression of RANKL, an indispensable factor in osteoclastogenesis (Yasuda, et al., 1999). From the staining results, the expression of the S1PR1-RANKL double-positive cells in the lesion tissues was confirmed, suggesting there might be a possible correlation between S1PR1 activation and RANKL production in apical periodontitis. These investigations on human periapical lesions revealed that S1P-S1PR1 signalling was aberrantly enhanced in

apical periodontitis and might take part in the pathogenesis of bone loss in apical periodontitis.

To further identify the role of S1P-S1PR1 signalling in the development of apical periodontitis, a rat model of this disease was created to find out the status of S1P-S1PR1 signalling during the pathological process and its relationship with osteoclastogenesis. In this study, periapical lesions were induced in rats, and the lesion size was measured using three methods: (1) through high-resolution X-ray imaging and (2) histological analyses to measure the area; (3) through μCT analysis to measure the volume. The results of X-ray imaging and histological analysis were identical. From the measurements of lesion area and volume, it could be observed that the periapical bone loss appeared and continued to expand between days 7 and 21 after pulp exposure; which became comparably stable after day 28. According to this, it could be concluded that the time between day 7 to day 21 is the active phase (rapid development of apical periodontitis), while that between day 28 to day 35 is the stable phase. These results agrees with the previous studies (Liu & Peng, 2013; Yang, et al., 2014), indicating that this model is reliable and effective for apical periodontitis research.

The results of immunohistochemical staining of rat mandibles revealed that the presence of $S1PR1^{+}$ cells (many of which were lymphocytes) persisted during the entire pathological process of apical periodontitis, accompanied with inflammatory cell infiltration. Compared with the control group, the expressions of S1PR1 were obviously much higher. These results indicated that the S1PR1 was activated during apical periodontitis, which could induce the migration of immune cells into inflammatory sites (Rivera, et al., 2008; Spiegel & Milstien, 2003). The cell counting results showed that the number of S1PR1-positive cells peaked on day 14 and then decreased during the stable phase (days 21 to 35). Of note, the numbers of osteoclasts and RANKL-positive cells exhibited a similar trend. The significant positive correlation between expression of S1PR1 and osteoclasts suggests that S1P-S1PR1 signalling takes part in the enhanced osteoclastogenesis during apical periodontitis. This might be partially due to the production of RANKL, as the RANKL expression was also positively correlated with S1PR1 expression.

To further verify the correlation between S1PR1 and RANKL, the double-dye immunofluorescent staining of S1PR1 and RANKL was also applied in different

stages of rat periapical lesion tissues. Same as in the human tissues, the $S1PR1^+$ $RANKL^+$ cells were observed in rat periapical lesion tissues, suggesting that the S1PR1 activation correlates with RANKL production in certain cells within the periapical lesions. Based on this, it could be presumed that the S1P-S1PR1 signalling participates in inducing osteoclastogenesis during apical periodontitis by enhancing RANKL production.

To confirm this presumption, a modulator on this signalling was applied in the rat periapical lesion model. The structural analogue of S1P–FTY720, which has been used in clinical treatment of multiple sclerosis (MS) (Kappos, et al., 2006), is found to reduce inflammation through its functional antagonistic effects on S1PR1 which making it unresponsive to S1P (Pham, et al., 2008; Thangada, et al., 2010); therefore resulting in inhibited differentiation, migration and functional maturation of immune cells. Thus, in this study, FTY720 treatment was used to negatively regulate the S1P-S1PR1 signalling in induced models of apical periodontitis in rats.

To evaluate the effectiveness of FTY720 treatment in apical periodontitis, firstly, its effect on bone loss was analysed. The lesion size was measured by histological analysis; the results showed that from day 14 to day 35, the periapical areas of experimental group were significantly smaller than those of control group, indicating that modulation of S1P-S1PR1 signalling with FTY720 effectively inhibited the alveolar bone destruction in apical periodontitis. This demonstrated that the S1P-S1PR1 signalling played a crucial role in apical periodontitis-derived bone loss.

Further experiments were conducted to investigate the mechanisms underlying this therapeutic effect. The results of TRAP analysis identified that the expression of osteoclasts was significantly down-regulated after day 14 following FTY720 administration, which effectively restrained osteoclastogenesis and kept it at a comparably low level. This finding, together with the previous experiments descried above, indicates that the S1P-S1PR1 signalling acts as a regulator in the osteoclastogenesis of apical periodontitis.

We then sought to figure out the impacts of modulating S1P-S1PR1 signalling on RANKL expression. As shown in Fig.8, the S1PR1 expression was significantly down-regulated by FTY720 during the whole pathogenesis of rat periapical lesions. Based on this result, it could be concluded that the S1PR1 expression was successfully reduced by FTY720 administration in this animal model. Following the down-

regulated S1PR1, RANKL expression was also significantly inhibited by FTY720 treatment, which stayed in a lower level compared to that of control group. This finding, together with the previous finding described above, further demonstrated that close correlation exits between S1PR1 and RANKL; that during the pathogenesis of apical periodontitis, S1PR1 activation induced by S1P could enhance the production of RANKL, therefore inducing the RANKL-mediated osteoclastogenesis. It is therefore speculated that the S1P-S1PR1-RANKL axis plays a central role in apical periodontitis-derived bone loss.

As the imbalance of RANKL/OPG was considered to be essential in bone destruction (Fonseca, et al., 2004), the OPG expression was investigated following modulation of the S1P-S1PR1 signalling by immunohistochemical staining. The results showed that there was no significant difference between the control and experiment groups; hence, the ratio of RANKL and OPG was obviously lower after FTY720 administration, indicating that FTY720 could attenuate the imbalance of RANKL/OPG in apical periodontitis. So far, S1P-S1PR1 signalling has not been reported to be related with OPG expression. Therefore, this finding suggested that the S1P-S1PR1 signalling up-regulated RANKL production while causing no effect on that of OPG. This induced the imbalance of RANKL/OPG, which results in activated bone resorption in apical periodontitis.

It should also be noted that the inhibition of S1P-S1PR1 signalling on bone loss was most effective during the active stage (day 7 to day 21) of apical periodontitis. When it came to the later stable stage (day 28 to day 35), the difference between the experimental and control groups became less obvious. As the S1P-S1PR1 signalling has also been reported to induce bone formation (Pederson, et al., 2008; Sato, et al., 2012), it could be assumed that down-regulation of this signalling may impact the bone formation in apical periodontitis. This indicates the role of S1P-S1PR1 signalling in the inflammatory bone remodelling is far more complex than expected and should be further addressed.

In summary, the results of the current study clearly demonstrate that the S1P-S1PR1 signalling plays a crucial role in the apical periodontitis, through enhancing RANKL production, inducing the imbalance of RANKL/OPG, and promoting osteoclastogenesis. The effective outcome of the inhibition of S1P-S1PR1 signalling indicates that this signalling could be a potential target for the treatment of apical

periodontitis. Our research findings also suggest that the administration of FTY720 should be considered as a potent therapeutic approach for treating apical periodontitis. It is generally accepted that root canal treatment is the best therapy for apical periodontitis, thus, FTY720 administration could be used as a supplementary treatment, especially for the acute apical periodontitis (due to its immunosuppressive functions). However, since humans are physically different from rats, the effect of FTY720 on human apical periodontitis still needs to be established in future studies.

Chapter 4:

Suggested Statement of Contribution of Co-Authors for Chapter by Published Paper

In the case of this chapter and half content of chapter 3

Title: **The role of S1P-S1PR1 signalling in the interaction between macrophages and BMSCs in infection-induced inflammation**

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Contributor	Statement of contribution
Lan Xiao	Involved in the conception and design of the project, performed laboratory experiments, data analysis and interpretation. Wrote the manuscript.
Yinghong Zhou	Involved in the conception and design of the project, data analysis and reviewed the manuscript.
Rong Huang	Assisted with data analysis.
Wei Shi	Assisted with sample collection and preparation.
Bin Peng	Involved in the conception and design of the project, and reviewed the manuscript.
Yin Xiao	Involved in the conception and design of the project, and reviewed the manuscript.

Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

Name: Prof Yin Xiao Signature: 

Date: 3rd March 2016

The role of S1P-S1PR1 signalling in the interaction between macrophages and BMSCs in infection-induced inflammation

4.1 ABSTRACT

Background: The S1P-S1PR1 signalling plays a crucial role in inducing bone loss during the infection-induced inflammation. It is still unclear what causes this activation and how this signalling leads to osteoclastogenesis. This study aims to investigate the role of S1P-S1PR1 signalling in macrophage-BMSC interaction under infection-induced inflammatory conditions.

Methods: The *in vitro* co-culture system of macrophages and BMSCs was applied with LPS stimulation to mimic the infection-induced inflammatory condition. S1PR1-siRNA was used to block S1PR1 in BMSCs co-cultured with macrophages. RT-qPCR, western blot and immunofluorescent analysis were performed to detect the expression levels of SPHK1, S1P, S1P1 and RANKL.

Results: Under LPS stimulation, BMSCs co-cultured with macrophages showed significant up-regulation of SPHK1 activity and S1P production, which then activated S1PR1 in BMSCs. This activated S1P-S1PR1 signalling eventually led to an increase of RANKL production by BMSCs, which was confirmed by S1PR1-blockage.

Conclusions: Under the infection-induced inflammatory conditions, macrophages were activated and interacted with BMSCs. This interaction induced the S1P production of BMSCs, which acted in an autocrine manner to activate S1PR1 of BMSCs. The activated S1P-S1PR1 signalling in BMSCs then induced the production of RANKL, therefore eventually resulted in enhanced osteoclastogenesis and bone loss.

4.2 INTRODUCTION

Bone is a dynamic tissue which is constantly undergoing life-long renovation. This renovation, also named remodelling, is constituted by osteoclast-derived bone resorption and osteoblast-derived bone formation (Raggatt & Partridge, 2010). Osteoclasts, derived from the monocyte/macrophage lineage, are so far recognised as the only type of cells capable of resorbing bone (Kini & Nandeesh, 2012). Osteoblasts, as well as the osteoblast precursors–BMSCs, not only direct the bone formation process, but also play a key role in osteoclastogenesis by producing receptor activator of RANKL (Udagawa, et al., 1990), which activates its receptor RANK on osteoclast-precursors and plays an indispensable role in osteoclastogenesis (Kong, et al., 1999; Theill, et al., 2002). The bone resorption and formation are kept in balance under physical circumstances (Arron & Choi, 2000). Under pathological settings such as inflammation, the balanced bone remodelling is disrupted; osteoclastogenesis is aberrantly activated leading to bone resorption exceeding bone formation, eventually results in bone loss, as seen in rheumatoid arthritis (RA) (Rodan & Martin, 2000), periodontitis (Taubman, et al., 2005), and apical periodontitis (Wang & Stashenko, 1993).

The balance of remodelling is highly regulated by the immune system (Takayanagi, 2007). The process of osteoclastogenesis is modulated by various immune-related factors, such as cytokines, transcription factors, receptors and molecular signalling (Takayanagi, 2005; Walsh et al., 2006). Among these factors, macrophages, which play a central role in the innate immune response, not only serve as precursors of osteoclasts, but also take part in the regulation of osteoclastogenesis (Takeshita, et al., 2000). Macrophages are a population of cells with three kinds of subsets: (1) non-activated M0 macrophages; (2) pro-inflammatory M1 macrophages which are classically activated by microbe-derived lipopolysaccharide (LPS) or Th1 cell-related cytokines such as IFNγ; and (3) M2 macrophages, which alternatively activated by Th2 cell-related cytokines such as IL-4 and IL-13, are identified as the anti-inflammatory macrophages (Horwood, 2015; Mantovani, et al., 2004; Mills, et al., 2000; Murray, et al., 2014). The M1 macrophage-derived cytokines either directly act on osteoclast-precursors (such as TNF-α and IL-1β) to induce osteoclastogenesis or indirectly act on osteoblasts (such as IL-6) or osteoblast-precursors (known as BMSCs) to promote RANKL production (Hashizume, et al., 2008; Kobayashi, et al., 2000;

Kudo, et al., 2003; Kurihara, et al., 1990; Lam, et al., 2000; Wei, et al., 2005; Zwerina, et al., 2007). Hence, the interaction between macrophages and osteoblast-lineage cells greatly affects RANKL production, therefore plays a central role in regulating osteoclastogenesis.

Several factors are found to be involved in the interaction between macrophages and BMSCs. According to recent research, S1P should also be considered as a potent coupling factor in the interaction between macrophages and BMSCs. S1P is the bioactive metabolite of sphingolipid (Maceyka, et al., 2012), which modulates diverse crucial cellular processes by binding with its five G protein-coupled receptors S1PR1 – S1PR5 (Davis & Kehrl, 2009); among which S1PR1 is widely expressed by various types of cells, and the S1P-S1PR1 signalling is found to play essential roles in osteoclastogenesis (Ishii, et al., 2009; Rivera, et al., 2008). A recent study (Ryu, et al., 2006) found that S1P production of osteoclast-precursors–known as macrophages/monocytes–was enhanced during osteoclastogenesis. The produced S1P then acted on osteoblast-precursors and induced the production of RANKL and therefore increasing the propensity for osteoclastogenesis. Activated S1PR1-S1PR3 were found in osteoblast-precursors, however, it is still unknown which receptor is responsible for enhancing RANKL production (Ryu, et al., 2006). Based on the previous findings, it could be speculated that macrophages interact with BMSCs through the S1P-S1PR1 signalling, which induces RANKL production and hence improve the propensity for osteoclastogenesis.

In the previous chapter, abnormally activated S1P-S1PR1 signalling in the infection-induced inflammation (apical periodontitis) has been identified, which is correlated with induced RANKL production and osteoclastogenesis. However, the driving mechanism for the activation of S1P-S1PR1 signalling is still not clear. Furthermore, it is still unknown how S1P induces RANKL production. Macrophages are activated during microbial-infection and play a central role in the immune response against pathogen invasion (Mantovani, et al., 2004), which interact with BMSCs to regulate osteoclastogenesis; this interaction might be greatly due to the S1P-S1PR1 activation. Hence, the aim of this study is to investigate the role of S1P-S1PR1 signalling in the macrophages-BMSCs interaction under the infection-induced inflammatory conditions.

4.3 MATERIALS AND METHODS

4.3.1 Cell culture

Rat BMSCs

BMSCs were obtained from 8-10 week old Wistar male rats (rBMSCs) as described in previous study (Leboy, Beresford, Devlin, & Owen, 1991). Briefly, the rats were sacrificed and the back limbs were then harvested. The femurs and tibias were dissected to remove all the skin and muscles, and then washed by phosphate buffered saline (PBS) with 1% (v/v) penicillin/streptomycin (P/S; Gibco®, Life Technologies Pty Ltd., Australia) for three times. The dissected bones were transferred into a 10 cm dish containing Dulbecco's modified Eagle's medium (DMEM; Gibco®, Life Technologies Pty Ltd., Australia). The two ends of the bones were cut open, and then the bone marrow was flushed into a 50 mL tube by syringes filled with DMEM (containing 1% P/S). The obtained bone marrow was then washed once with PBS (containing 1% P/S) and resuspended in DMEM containing 10% (v/v) fetal bovine serum (FBS; In Vitro Technologies, Australia) and 1% P/S. The cell suspension was then transferred into a culture flask and cultured in incubators with 5% CO_2 at 37º C. After three days, the non-adherent cells were washed away by PBS. The adherent cells were then continually incubated in DMEM with 10% FBS and 1% P/S (culture medium was changed every 2-3 days). When reaching 80% confluence, the cells were passaged by treating with 0.25% trypsin (containing 1mM EDTA, Gibco®, Life Technologies Pty Ltd., Australia) for 2 min. Cells within 5 passages were used for experiment. All experiment procedures were approved by the Ethics Committee of Queensland University of Technology.

RAW 264.7 cells

In this study, a mouse-derived macrophage cell line, RAW 264.7 (RAW) cells, were used as macrophage-like cells to study the interaction between macrophages and BMSCs. As a murine macrophage cell line, RAW cells have been proved to work reliably with BMSCs from other species (Chen, Wu, Gu, et al., 2014; Chen, Wu, Yuen, et al., 2014; Shi et al., 2016). RAW cells were cultured in DMEM with 10% FBS (heat-inactivated at 60 º C for over 30 min) and 1% P/S. The culture medium was changed every 2 to 3 days. After reaching 80% confluence, the cells were passaged by treating with 0.25% trypsin (containing 1mM EDTA) for 2 min.

4.3.2 *In vitro* cell co-culture model

LPS stimulation was used to simulate the infection-induced inflammatory condition as previously described (King, Fleming, Critchley, & Kelly, 2002). The rBMSCs were co-cultured with RAW cells under normal or infection-induced inflammatory conditions. Two co-culture models were established as follows:

Co-culture model One

To obtain conditioned medium (CM) of RAW cells from LPS stimulation, RAW cells were cultured in T175 flask to reach confluence. The cells were then stimulated with 100 ng/mL LPS for 12 h. After stimulation, the medium was removed, and the cells were washed twice with PBS, and then incubated with serum-free DMEM for 12 h. The medium was harvested and subjected to centrifugation (1000$\times g$, 10 min, 4 ºC), then filtered with a 0.2 μm filter (Millipore Corporation, Billerica, MA, USA) to remove cell debris. The filtered medium was stored at -80 ºC for further experiments.

To mimic normal condition as comparison, the CM from RAW cells (without LPS stimulation) was mixed with culture medium (DMEM, 20% FBS, 2% P/S) in a ratio of 1:1. To mimic inflammatory condition, the CM from LPS-stimulated RAW cells was mixed with culture medium containing 200 ng/mL LPS (DMEM, 20% FBS, 2% P/S) a 1:1 ratio. The mixed medium was then applied to rBMSCs. The rBMSCs in normal culture medium served as controls for the normal co-culture, while the ones in culture medium supplemented with 100 ng/mL LPS served as control for the LPS-induced inflammatory co-culture (Fig 19). The cells were stimulated for 12 h, and then harvested for RNA/protein extraction and immunofluorescent staining.

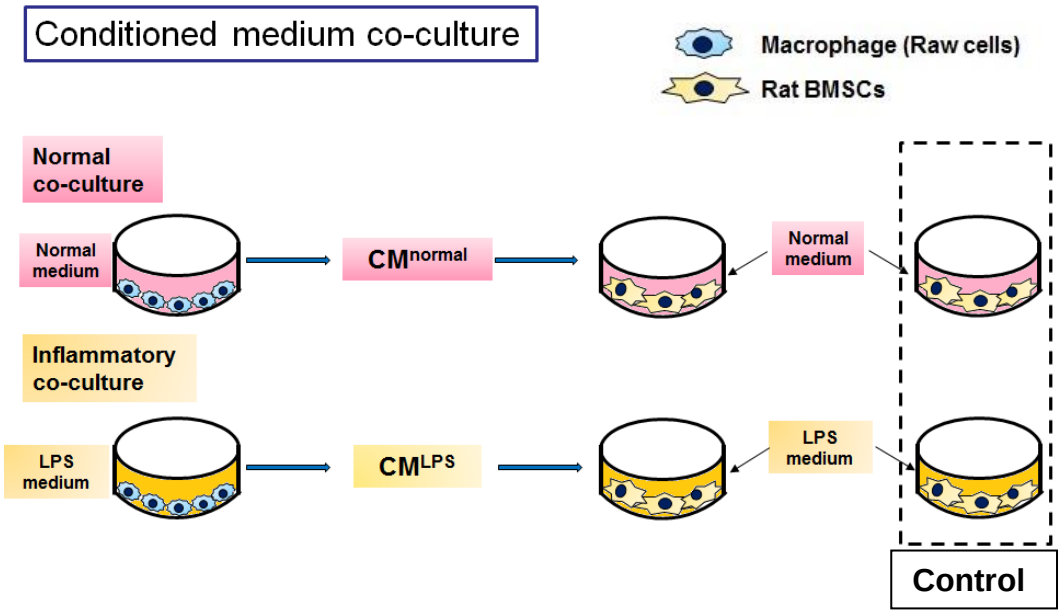

Figure 19. Co-culture model (conditioned medium) of macrophages and rBMSCs. CM of macrophages under normal or LPS stimulation was collected and mixed with normal or LPS medium; the mixed medium was then applied to rBMSCs. The rBMSCs applied with normal or LPS medium were served as normal- or LPS-controls, respectively. CM = conditioned medium.

Co-culture model Two

A trans-well co-culture system was used to further monitor the interaction between macrophages and rBMSCs. The cell culture inserts for 6-well plates (BectonDickinson Labware, Franklin Lakes, NJ, USA) with 0.4 µm pore size were used to culture RAW cells. The rBMSCs were seeded onto the companion plates (the ratio of numbers of rBMSCs to RAW cells is roughly 1:4). After 24 h of attachment, the inserts were then assembled onto the companion plates. For normal co-culture, normal culture medium (supplemented with FBS inactivated) was applied to the inserts and companion plates. The singly cultured rBMSCs with normal culture medium served as normal controls. In the LPS-induced inflammatory condition, co-cultured cells were stimulated with 100 ng/mL LPS (in DMEM with 10% inactivated FBS and 1% P/S), the singly cultured rBMSCs subjected to the same medium served as LPS-controls (Fig 20). After 12 h of co-culture, the cells were harvested for subsequent experiments.

4.3.3 S1PR1 siRNA Transfection

The S1PR1 siRNA (5'GAC UAU GGC AAC UAU GAU A3', 5'UAU CAU AGU UGC CAU AGU C3', Product number: PDSIRNA2D, siRNA ID: SASI_Rn01_00101785, Sigma-Aldrich Pty. Ltd., Sydney, Australia) was used to block S1PR1 in this study. The experiment was performed following the manufacturer's instructions. The rBMSCs for transfection were seeded in 6-well plates with culture medium (DMEM, 10% FBS, no P/S). After 24 h of cell attachment, forward transfection of S1PR1 siRNA was performed on each well. 125 pmol S1PR1 siRNA was diluted in 250 µL Opti-MEM® I Reduced Serum Medium (Opti-MEM, Gibco®, Life Technologies Pty Ltd., Australia). Also, 4 µL Lipofectamine™ RNAiMAX was diluted in the same amount of Opti-MEM. These two dilutions were gently mixed and incubated for 15 min at room temperature, and then transferred into the well. The cells were then incubated for 48 h in the incubator. After incubation, the rBMSCs were then co-cultured with RAW cells using the trans-well system as described above (Fig 20). The universal negative control siRNA (MISSION® siRNA Universal Negative Control #1, Product number: SIC001, Sigma-Aldrich Pty Ltd., Sydney, Australia) was used in wells without the application of S1PR1 siRNA.

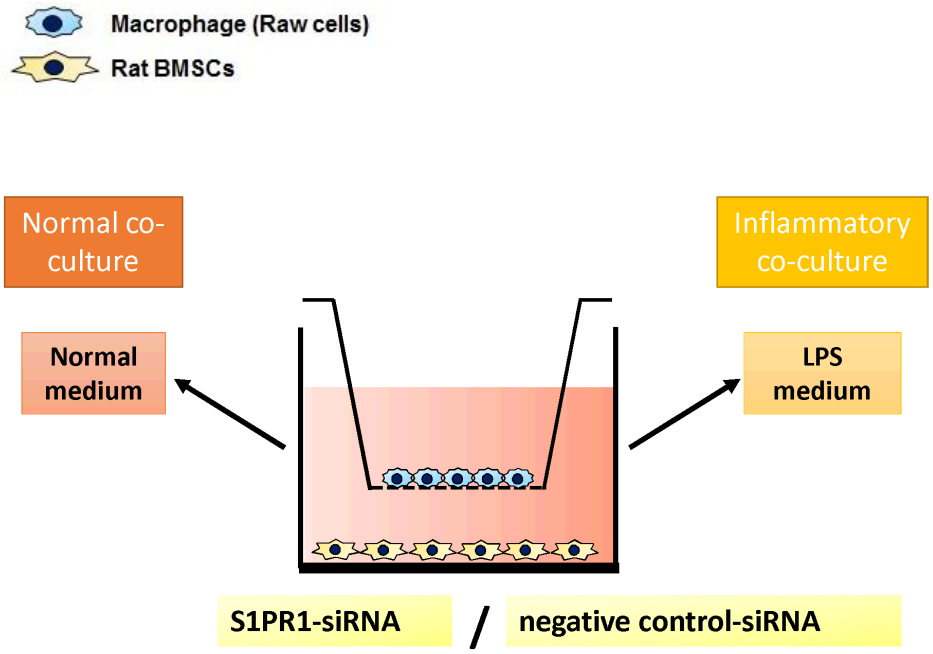

Figure 20. Co-culture model (trans-well) of macrophages and rBMSCs. S1PR1 siRNA was used for blockage of S1PR1, while negative control siRNA was used in wells without the application of

S1PR1 siRNA. RBMSCs singly cultured in normal/LPS medium were used as controls for normal or inflammatory co-culture, respectively.

4.3.4 RNA extraction, cDNA synthesis, and real time quantitative-PCR (RT-qPCR)

Total RNA was extracted from rBMSCs or RAW cells using the TRIzol Reagent (Ambion®, Life Technologies Pty Ltd., Australia). The cDNA was then synthesized from 1 μg total RNA by SensiFAST™ cDNA Synthesis Kit (Bioline (Aust) Pty Ltd., Australia) following the manufacturer's protocol. The real-time polymerase chain reaction (RT-qPCR) was carried out by the QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Australia) with SYBR® Green reagent (Applied Biosystems, Australia) according to the manufacturer's instructions. The RT-qPCR primers (Table 4) were designed based on cDNA sequences from the National Centre for Biotechnology Information (NCBI) sequence database and the primer specificity was confirmed by Primer-BLAST on the NCBI website. Analyses were performed on the following target genes: *SPHK1*, *S1PR1*, and *TNFS11* (rat); *CD86*, *TNF*, *iNOS*, *IL-6*, *IL-1β, CCR7, SPHK1* and *S1PR1* (mice). The house keeping gene *18S* and *β-actin* were used as control. All reactions were run in triplicate for three independent experiments. Relative gene expression was normalized against *18S* or *β-actin* and calculated as previously described (Bookout & Mangelsdorf, 2003).

Table 4. Primer sequences for the gene investigated in this study

Rat

Gene	Forward sequences	Reverse sequences
SPHK1	5'AAGCTGGGCTGTCCTTCAAC3'	5'ACAGCGTCTTCCCAATCTGG3'
S1PR1	5'GTTGTCCGGGATTTGGTAGG3'	5'GATGCTCGTAGGGGTTAGAG3'
TNFS11	5'GGCATCATGAAACCTCAGGG3'	5'GTTGGACACCTGGACGCTAA3'
18S	5'CGGAACTGAGGCCATGATTAAG3'	5'GTATCTGATCGTCTTCGAACCTCC3
β-actin	5'ATGCAGCCTGAAGAGGACTG3'	5'GGCTATGAAATCCAGGGCCT3'

Mice

Gene	Forward sequences	Reverse sequences
SPHK1	5'AGAGTGCTGGTGCTGCTGAA3'	5'CATGCATCAGACCATCACCG3'
S1PR1	5'ACTTTGCGAGTGAGCTGGTC3'	5'AGGAGCCTGGGGTGGTATTT3'
CD86	5'CTGCTCATCATTGTATGTCAC3'	5'ACTGCCTTCACTCTGCATTTG3'
TNF	5'CTGAACTTCGGGGTGATCGG3'	5'GGCTTGTCACTCGAATTTTGAGA3'
iNOS	5'TGGTGAAGGGACTGAGCTGT3'	5'CTGAGAACAGCACAAGGGGT3'
IL-6	5'GTCTTCTGGAGTACCATAGCTACCTG3'	5'CCTTCTGTGACTCCAGCTTATCTG3'
IL-1β	5'TGGAGAGTGTGGATCCCAAG3'	5'GGTGCTGATGTACCAGTTGG3'
CCR7	5'ATGACGTCACCTACAGCCTG3'	5'CAGCCCAAGTCCTTGAAGAG3'
18S	5'CGGAACTGAGGCCATGATTAAG3'	5'GTATCTGATCGTCTTCGAACCTCC3
β-actin	5'ACTGAGCGTGGCTATTCCTTCG3'	5'CTAGGGCCGTGATCTCCTTCTG3'

4.3.5 Protein extraction and western blotting

Total protein was extracted from rBMSCs by the lysis buffer (20 mM HEPES (pH 7.4), 10% glycerol, 1% Triton X-100, 2mM EDTA) with the protease inhibitor cocktail (Roche Products Pty. Ltd., Dee Why, NSW, Australia). Measurements of protein concentration were performed by using the BCA Protein Assay Kit (Thermo Fisher Scientific, VIC, Australia). For each sample, 10 μg of protein was loaded into a SDS-PAGE gel and then separated by running the gel. After that the protein was transferred to a nitrocellulose membrane (Merck Millipore, Billerica, USA). The membrane was blocked by the Odessy buffer (LI-COR Biosciences, Lincoln, USA) for 1 h at room temperature and then incubated with primary antibodies against SPHK1 (1: 500, Abcam, Cambridge, UK), S1P (1: 800, Abcam, Cambridge, UK), S1PR1 (1: 500, Abcam, Cambridge, UK), and RANKL (1:300, Novus Biologicals, LLC, USA); α-Tubulin (1: 5000, Abcam, Cambridge, UK) was used as a loading control. After incubated at 4 ℃ overnight, the membrane was washed and then incubated with Anti-

rabbit IgG IRDye 800 conjugated secondary antibody (1: 10000, Rockland, Gilbertsville, Pennsylvania, USA) or Anti-mouse IgG IRDye 700 conjugated secondary antibody (1: 4000, Rockland, Gilbertsville, Pennsylvania, USA) for 1 h at room temperature. All the antibodies were diluted in the Odessy buffer. After three-times of washing, the membranes were scanned by Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, USA) according to the manufacturer's instructions. Quantification of band intensities was obtained by ImageJ software. All experiments were replicated by three times.

4.3.6 Immunofluorescent staining

The rMSCs were seeded on 13mm coverslips (ProSciTech, QLD, Australia) for immunofluorescent staining. After treatment (described in sections 4.3.5 and 4.3.6), the rBMSCs were washed twice by PBS, and then fixed by 4% PFA for 20 min at room temperature. The cells were washed three times by PBS, and then permeabilized by 0.1% Triton X-100 (diluted in PBS) for 5 min at room temperature. After washing (three times in PBS), the cells were blocked in 1% BSA/PBS (60 min in room temperature), then incubated with primary antibodies against SPHK1 (1: 150), S1P (1: 200), S1PR1 (1: 100), and RANKL (1: 100) at 4°C overnight. Then the cells were washed three times by 0.1% BSA/PBS (5 min each time) and then incubated with secondary antibodies, goat anti-rabbit Alexa Fluor® 488 (1:1000, Cell Signaling Technology, Inc, Danvers, USA), goat anti-mouse Alexa Fluor® 568 (1:1000, Life Technologies Pty Ltd., Australia)for 30min (protected from light). All antibodies were diluted in 1% BSA/PBS. After three times' washing (0.1% BSA/PBS, 5 min), the cells were mounted with ProLong® Gold antifade reagent with DAPI (Life Technologies Pty Ltd., Australia), secured to glass slides, kept at 4℃ overnight, and then analysed under the fluorescent microscope. In each section, S1P-positive cells/S1PR1-positive cells/SPHK1-positive cells/RANKL-positive cells/S1PR1-RANKL double-positive cells in five randomly selected regions were counted under 200× magnification; the percentage of positive cells in total cells was calculated. The average percentage of the five chosen areas was calculated. Three sections from each group were analysed for cell measurements. All measurements were performed in a double-blind manner by two trained independent observers.

4.3.7 Statistical analysis

All data were subjected to statistical analysis using one-way ANOVA, followed by the Student-Newman-Keul test at a = 0.05. Pearson correlation was used for correlation analysis on numbers of $S1PR1^+$ cells with osteoclasts and $RANKL^+$ cells (a = 0.05). A $p< 0.05$ was considered to significantly different. Data were analysed by SPSS 13.0 (SPSS Inc., Chicago, IL). All data were presented as mean ± standard deviation (SD).

4.4 RESULTS

4.4.1 SPHK1 and S1PR1 were down-regulated in macrophages stimulated by LPS, while up-regulated in rBMSCs stimulated by macrophage CM

In this study, firstly, the mRNA levels of SPHK1 and S1PR1 were examined in RAW cells stimulated with LPS. The macrophage phenotype was also analysed by RT-qPCR. The mRNA expression levels of typical markers of M1 macrophages – TNF-α, IL-1β, iNOS, IL-6, CCR7 and CD86, were found to be significantly enhanced in RAW cells (Fig 21), indicating the macrophages were polarized towards the M1 phenotype by LPS stimulation. This is consistent with previous studies (Horwood, 2015; Mantovani, et al., 2004; Mosser & Edwards, 2008; Tjiu, et al., 2009). Along with the increased expression of M1 markers, there was a significant decrease in the expression of SPHK1. Furthermore, decreased S1PR1 expression was observed. This suggested that S1P production of macrophages was reduced when the cells were stimulated by LPS; hence, macrophages would not likely be the major source of S1P in the infectious inflammatory condition.

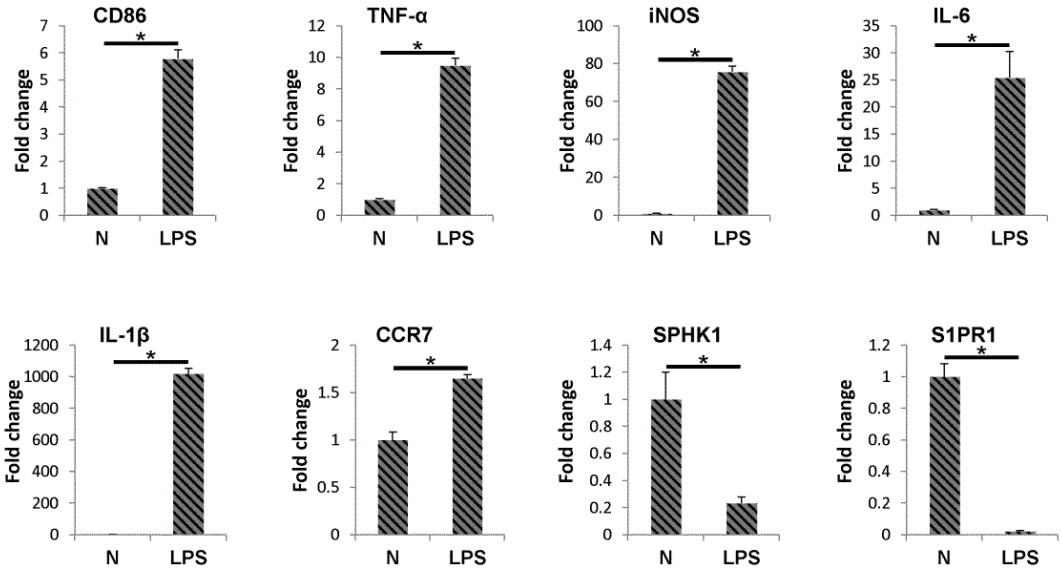

Figure 21. Changes of mRNA levels in macrophages under the stimulation of LPS (n=3). The mRNA levels of M1 macrophage markers (CD86, TNF-α, iNOS, IL-6, IL-1β and CCR7) were significantly increased in macrophages (RAW cells) stimulated by LPS; while the levels of SPHK1 and S1PR1 were significantly decreased (*p< 0.05), suggesting that S1P production was decreased in macrophages stimulated with LPS. N = normal medium. LPS = medium with LPS.

Next, the mRNA expression levels of SPHK1 and S1PR1 were examined in rBMSCs stimulated with CM derived from RAW cells. As shown in Fig 22, the SPHK1 expression was enhanced in rBMSCs stimulated with macrophage CM under both normal and LPS-induced inflammatory conditions; accordingly, the S1PR1 expression of rBMSCs was also up-regulated when exposed to macrophage CM. It could also be observed that the SPHK1 and S1PR1 expression was significantly higher in CM-treated rBMSCs under the stimulation of LPS. This suggested that the secreted factors from macrophages induced SPHK1 expression in rBMSCs, especially under the infection-induced inflammatory condition.

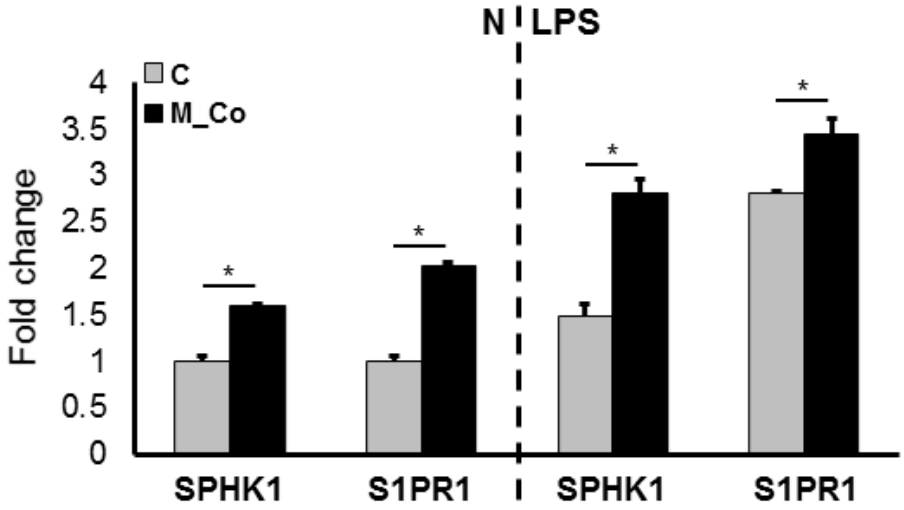

Figure 22. Change of mRNA levels in rBMSCs stimulated by CM derived from macrophages (n=3). Under both normal and LPS-stimulation conditions, the mRNA levels of SPHK1 and S1PR1 in rBMSCs were significantly up-regulated when stimulated by CM and macrophages (*P < .05), (rBMSCs in normal culture medium served as controls for the normal co-culture, while the ones in culture medium supplemented with LPS served as control for the LPS-induced inflammatory co-culture). N = normal medium. LPS = medium with LPS. CM = conditioned medium from RAW cells with/without LPS stimulation. M0 = M0 macrophage. M1 = M1 macrophage.

4.4.2 Up-regulated SPHK1-S1P-S1PR1 axis and RANKL production in rBMSCs co-cultured with macrophages

To further study the interaction between macrophages and BMSCs, these two kinds of cells were co-cultured by using the trans-well system. From the Result 4.4.1, it could be concluded that the rBMSCs should be the main source of S1P in the co-culture under the infectious inflammatory conditions; hence, in the following study,

we only focused on analysing rBMSCs in the co-culture. The RT-qPCR result showed that, when co-cultured with macrophages (using the transwell co-culture system) in normal condition, the rBMSCs showed a slight increase in; while in the LPS-induced inflammatory condition, co-culturing with macrophages significantly induced SPHK1 mRNA levels (Fig 23); this was consistent with the results of western blot (Fig 23) and immunofluorescent staining (Fig 24). Accordingly, the expression of S1P was up-regulated in rBMSCs co-cultured with macrophages, especially under LPS stimulation (Fig 23, Fig 25). These results indicated that the SPHK1 activity of rBMSCs was activated when co-culturing macrophages, which resulted in enhanced production of S1P; especially under the infection-induced inflammatory condition.

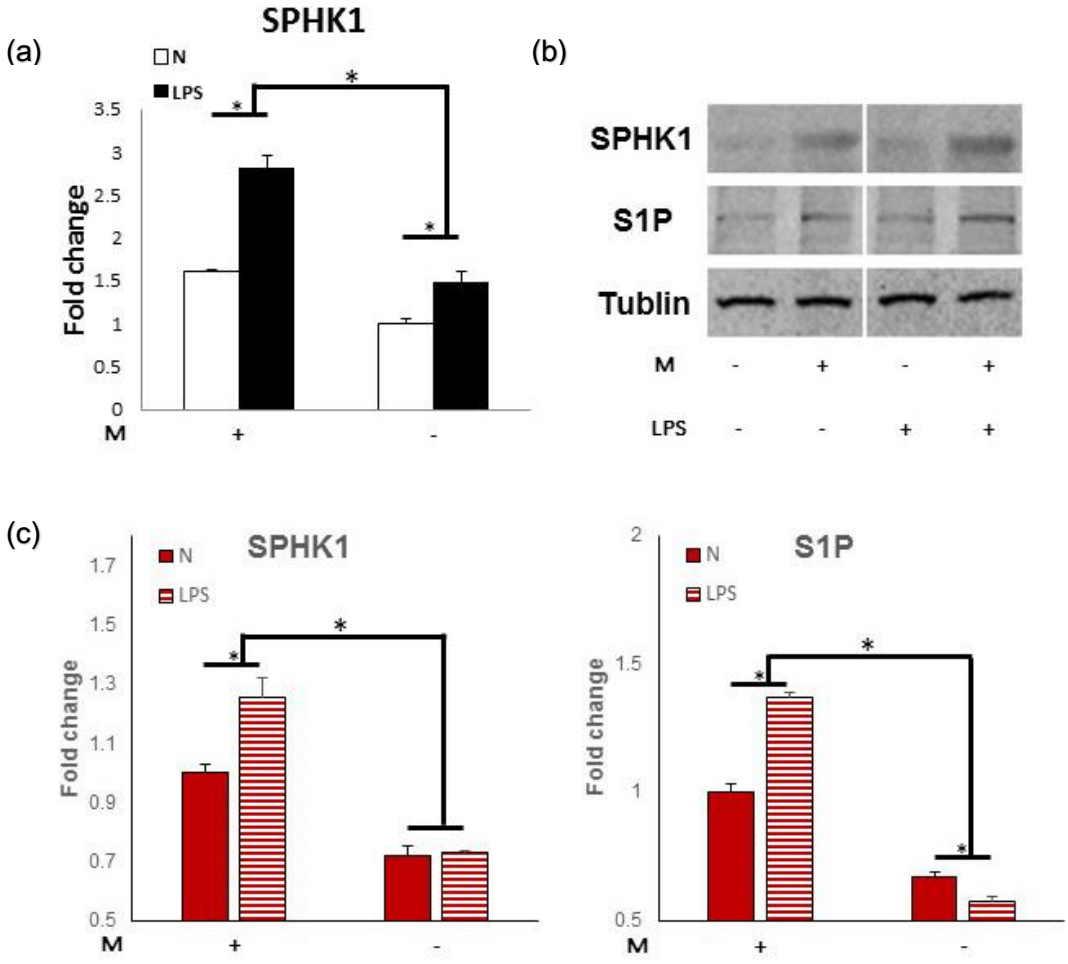

Figure 23. Up-regulated SPHK1 and S1P in rBMSCs co-cultured with macrophages (n=3). (a) Under both normal and LPS-stimulation conditions, the mRNA level of SPHK1 in rBMSCs was significantly increased when co-cultured with macrophages (*p< 0.05). (b) The protein levels of SPHK1 and S1P were up-regulated in rBMSCs co-cultured with macrophages under both normal and LPS-stimulation conditions. (c) Quantification of band intensities showed the protein levels of SPHK1 and S1P were significantly higher in rBMSCs co-cultured with macrophages (*p< 0.05), suggesting that macrophages induced the SPHK1 activity and hence S1P production in the co-cultured rBMSCs,

especially under the infectious inflammatory condition. M = co-cultured with macrophages, N = normal medium, LPS = medium with LPS.

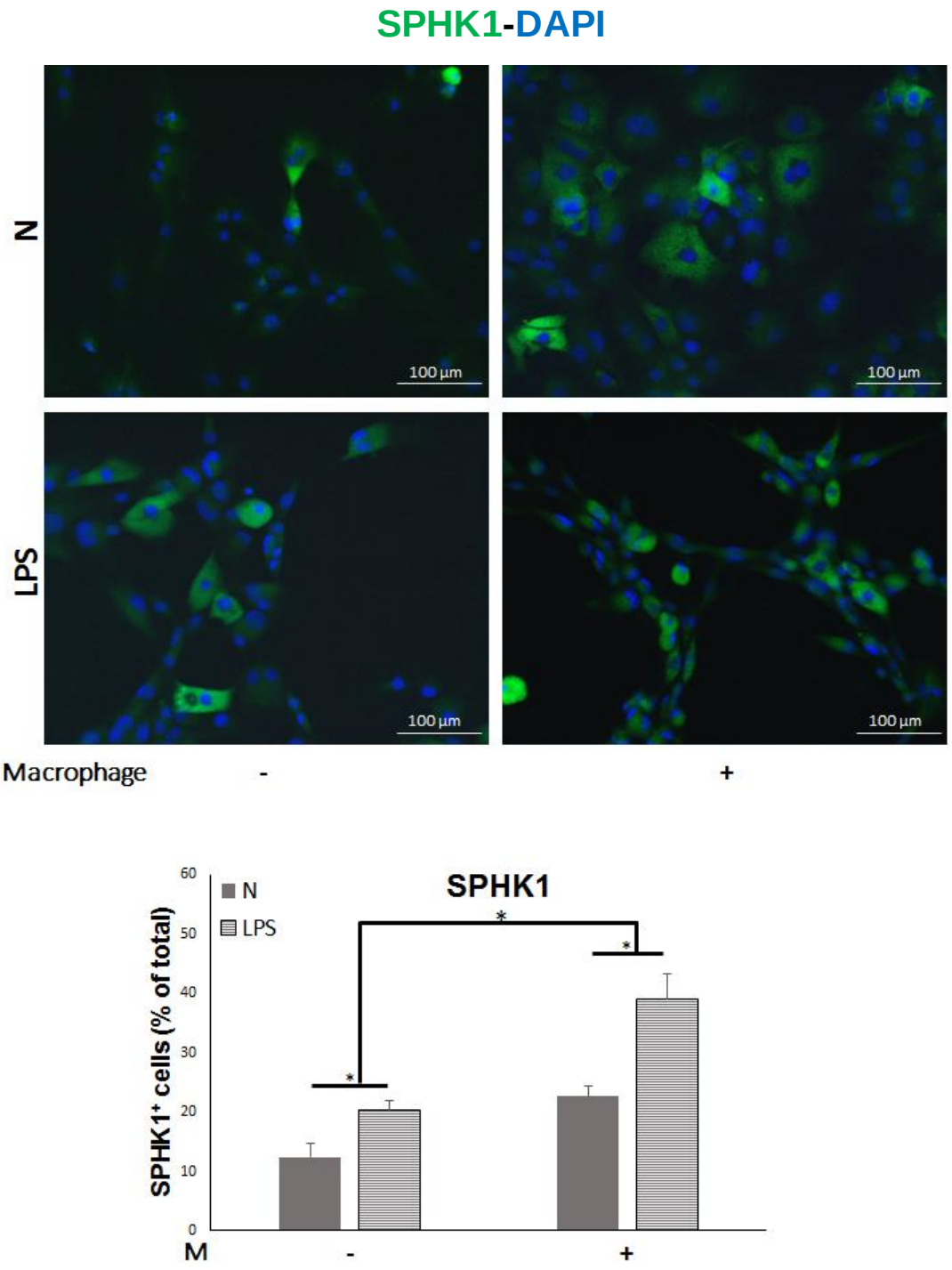

Figure 24. Up-regulated SPHK1 in rBMSCs co-cultured with macrophages (original magnification, 200×, Scale bar = 100 µm, n=3). It could be observed that under both normal and LPS-stimulation conditions, there were more SPHK1-positive cells in rBMSCs co-cultured with macrophages (*p< 0.05); the cell counting results indicated that the SPHK1 expression was significantly higher in rBMSCs co-cultured with macrophages, especially under infectious inflammatory conditions. Macrophage & M = co-cultured with macrophages, LPS = medium with LPS.

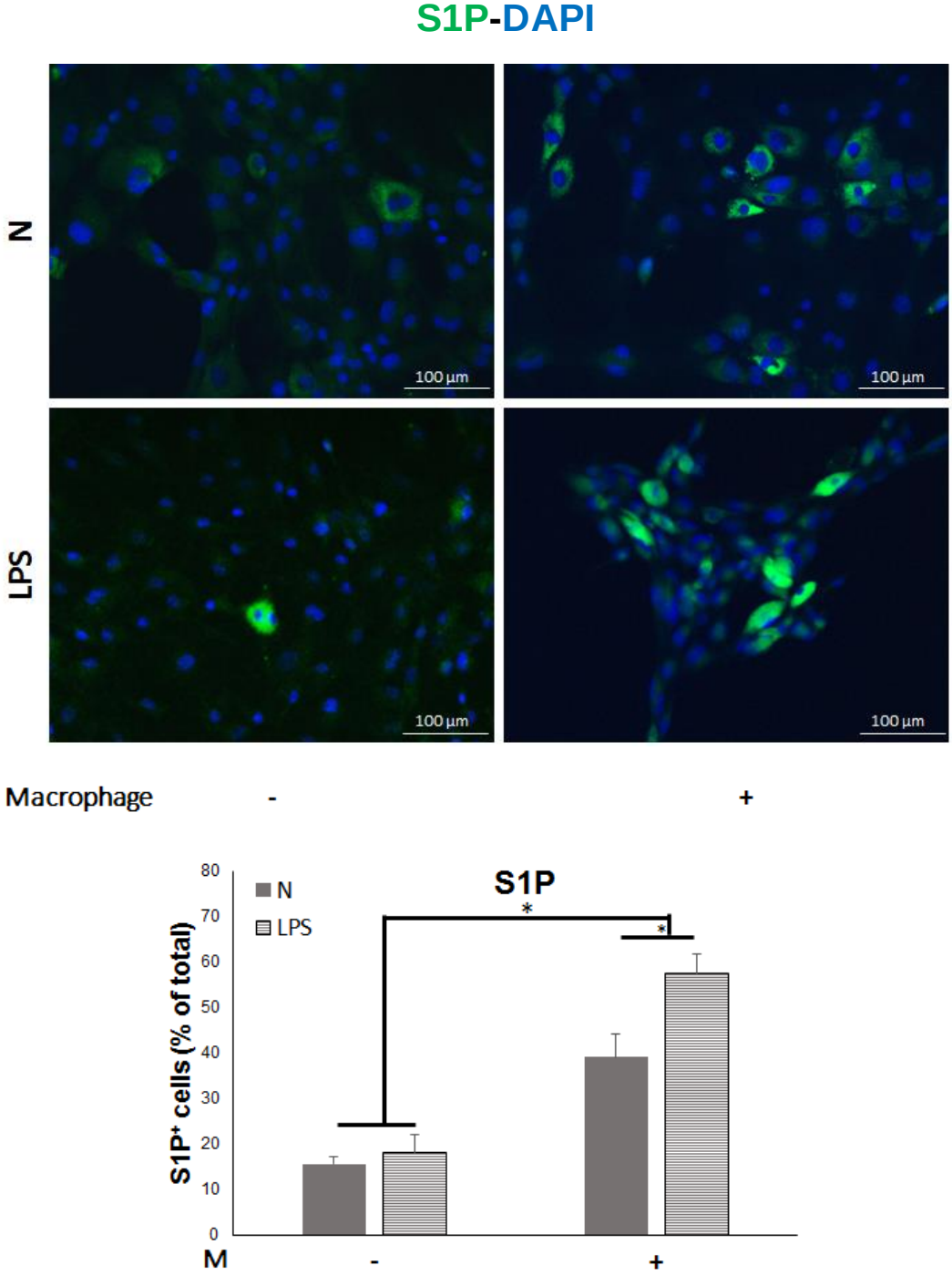

Figure 25. Up-regulated S1P in rBMSCs co-cultured with macrophages (original magnification, 200×, Scale bar = 100 µm, n=3). It could be observed that under both normal and LPS-stimulation conditions, there were more S1P-positive cells in rBMSCs co-cultured with macrophages (*p< 0.05); the cell counting results indicated that the S1P expression was significantly higher in rBMSCs co-cultured with macrophages, especially under infectious inflammatory conditions.. Macrophage & M = co-cultured with macrophages, LPS = medium with LPS.

As S1P production was found to be increased, the status of S1PR1 and RANKL was then examined in the co-cultured rBMSCs. Through the results of RT-qPCR and western blot, it could be observed that the expression of S1PR1 was significantly up-regulated along with the activated SPHK1 and increased S1P production. Similar to S1PR1, the expression of RANKL was found to be up-regulated in rBMSCs co-cultured with macrophages, especially with the stimulation of LPS (Fig 26). This further confirmed that in infection-induced inflammatory microenvironments, secreted factors from macrophages induced the SPHK1 activity in rBMSCs, therefore activating the S1P-S1PR1 signalling. Also, the accordingly increased RANKL production suggested that the S1P-S1PR1 signalling might be due to RANKL synthesis in rBMSCs.

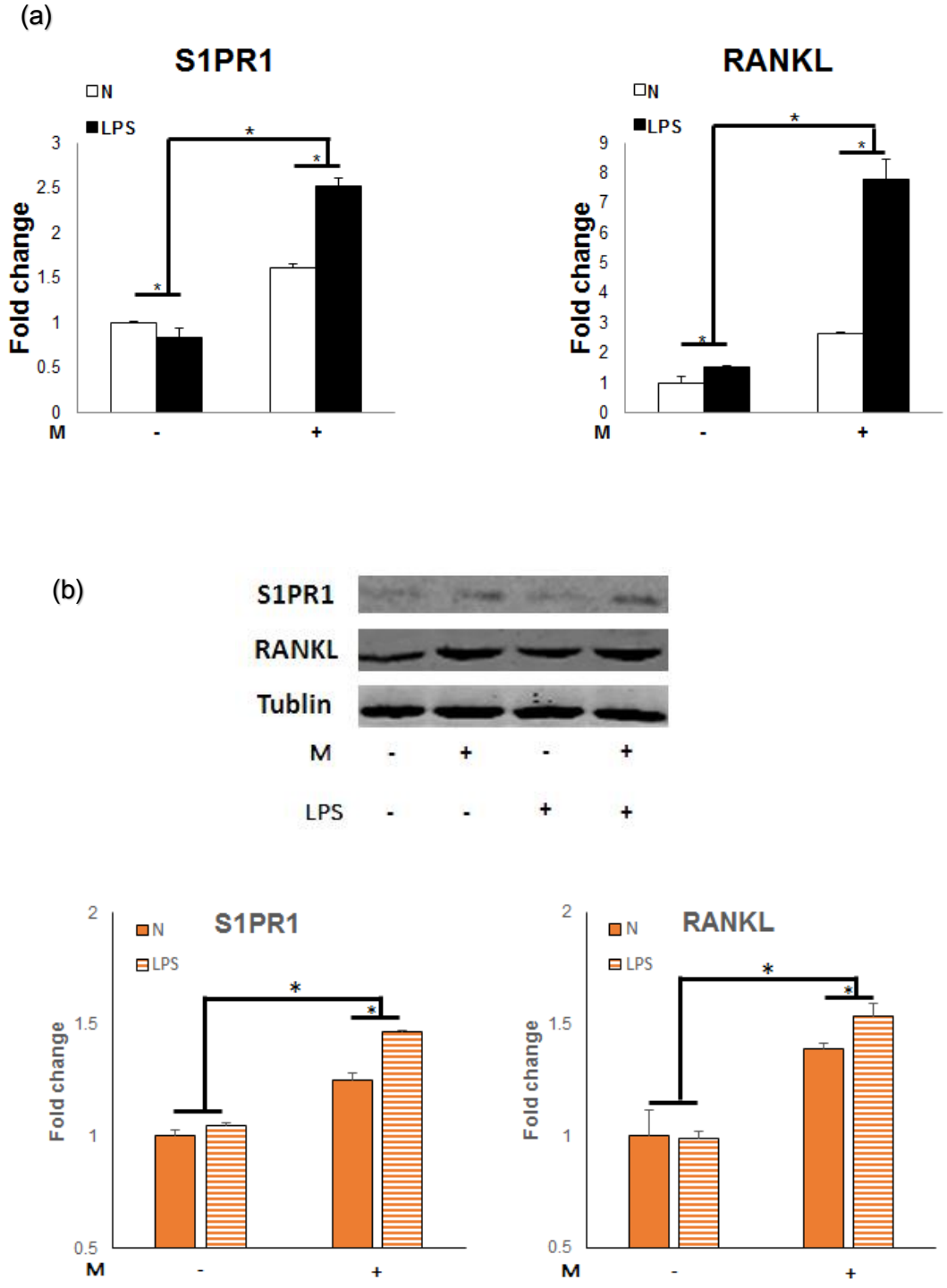

Figure 26. Up-regulated S1PR1 and RANKL in rBMSCs co-cultured with macrophages (n=3). (a) The RT-qPCR results showed that under both normal and LPS-stimulation conditions, the mRNA levels of S1PR1 and RANKL in rBMSCs were significantly up-regulated when co-cultured with macrophages (*p<0.05). (b) From the western blot results, it could be found that the protein levels of S1PR1 and RANKL were significantly increased in rBMSCs co-cultured with macrophages under both normal and LPS-stimulation conditions. M = co-cultured with macrophages, N = normal medium, LPS = medium with LPS.

4.4.3 The S1P-S1PR1 signalling regulated RANKL production in rBMSCs co-cultured with macrophages

To figure out whether S1PR1 acted as a regulator for rBMSCs RANKL production in the co-culture system, S1PR1 activity was blocked in rBMSCs using S1PR1 siRNA. As shown in Fig 27, S1PR1 siRNA successfully down-regulated the mRNA and protein expression of S1PR1. Accordingly, the RANKL expression decreased in rBMSCs with S1PR1 blockage. The double-dye immunofluorescent staining of S1PR1 and RANKL further confirmed the correlation of S1PR1 and RANKL in rBMSCs; as shown in Fig 28, $S1PR1^{+}RANKL^{+}$ cells were found in rBMSCs co-cultured with macrophages, especially when stimulated by LPS. The numbers of $S1PR1^{+}RANKL^{+}$ cells, as well as the $RANKL^{+}$ cells were decreased when S1PR1 was inactivated. This indicated that the S1P-S1PR1 signalling was responsible for the enhanced RANKL production in rBMSCs.

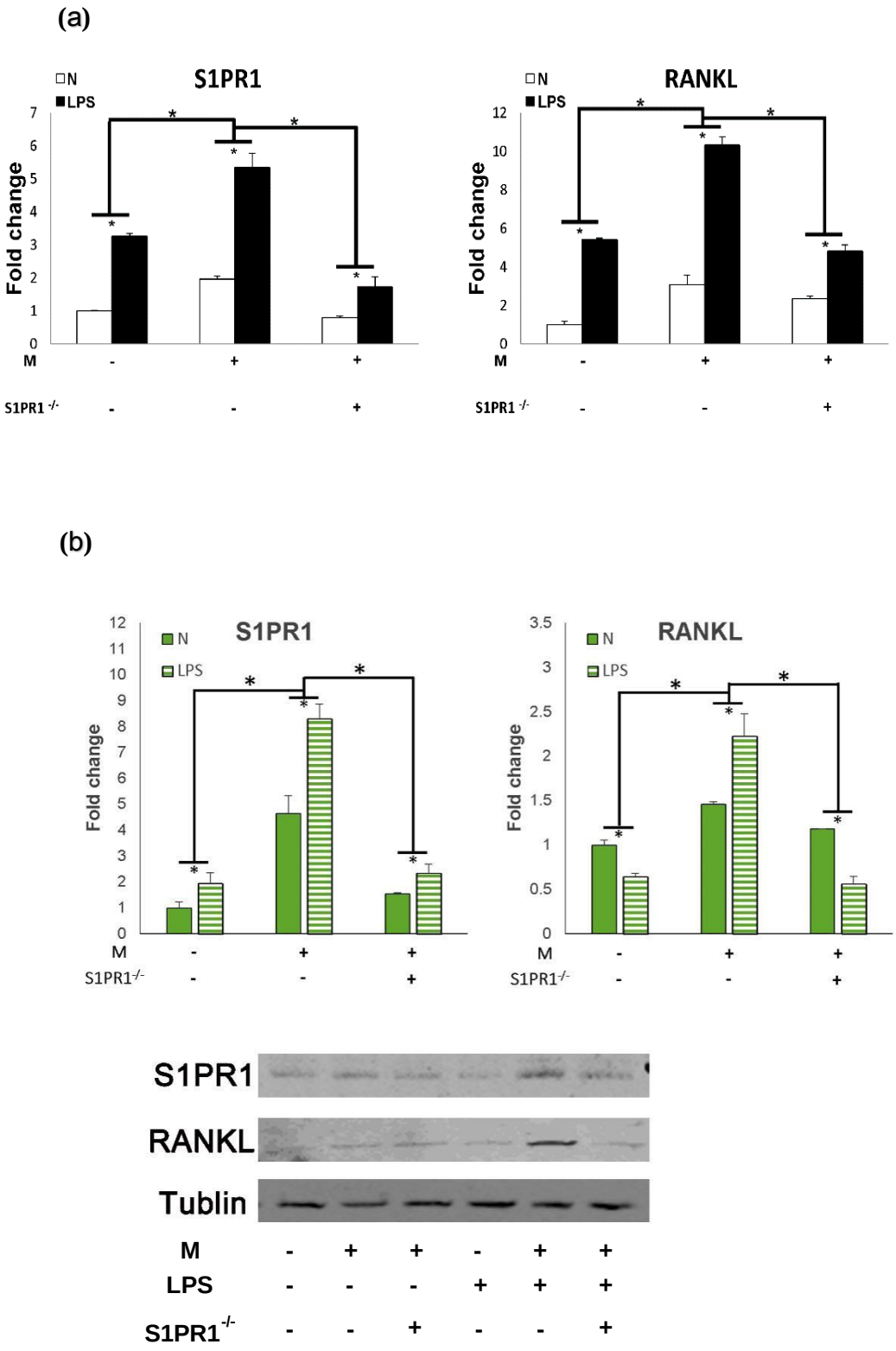

Figure 27. S1PR1 acted as the regulator for RANKL production of rBMSCs co-cultured with macrophages (n=3). (a) Under both normal and LPS-stimulation conditions, S1PR1-blockage led to decreased RANKL mRNA levels in rBMSCs co-cultured with macrophages (*p< 0.05). (b) The protein levels of RANKL were down-regulated by S1PR1 blockage in rBMSCs co-cultured with macrophages under both normal and LPS-stimulation conditions (*p< 0.05). M = co-cultured with macrophages, N = normal medium, LPS = medium with LPS, S1PR1 $^{-/-}$ = S1PR1 siRNA application.

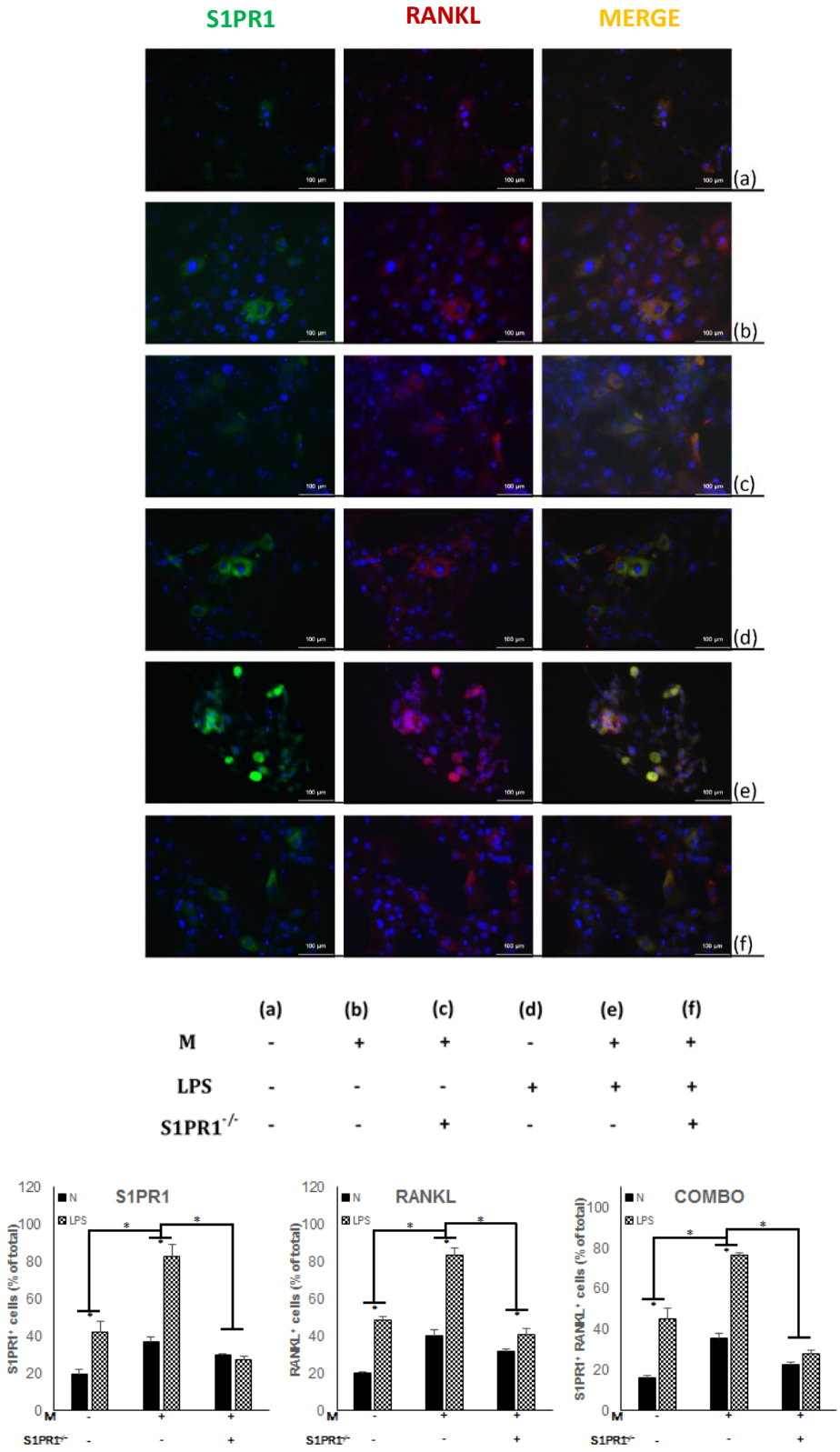

Figure 28. S1PR1 acted as the regulator for RANKL production of rBMSCs co-cultured with macrophages (n=3). Under both normal and LPS-stimulation conditions, there were more $S1PR1^{+}RANKL^{+}$ cells in rBMSCs co-cultured with macrophages; the $RANKL^{+}$ and $S1PR1^{+}$ $RANKL^{+}$ cells were significantly decreased in rBMSCs when S1PR1 was inactivated (original magnification, 200×, Scale bar = 100 μm) (*p< 0.05). M = co-cultured with macrophages, LPS = medium with LPS, S1PR1 -/- = S1PR1 siRNA application.

4.5 DISCUSSION

In this study, to investigate the interaction between macrophages and BMSCs, co-culture systems were established under normal or infection-induced inflammatory conditions. LPS was used to mimic infection induced inflammation as previously described (King, et al., 2002). Firstly, the possible effect of macrophage-derived secreted factors on rBMSCs was examined. To modulate the co-culture under normal condition or infectious inflammation, M0 macrophages were stimulated with/without LPS. In the normal co-culture group, the CM derived from macrophages without LPS stimulation was applied to rBMSCs cultured in normal culture medium; while in the inflammation group, the CM derived from LPS activated macrophages was applied to rBMSCs cultured in medium containing LPS.

The phenotype of macrophages was examined by RT-qPCR. The results showed that the M0 macrophages differentiated into typical M1 macrophages under the stimulation of LPS, which was consistent with previous studies (Mosser & Edwards, 2008). It was then found out that when compared with M0 macrophages, the SPHK1 expression was significantly down-regulated in M1 macrophages, indicating that in macrophages, the ability of S1P production reduced when they differentiated into the M1 phenotype. Surprisingly, significantly activated S1PR1 was found in rBMSCs applied with the M1 macrophage CM, suggesting that rBMSCs might be the source of S1P. The SPHK1 expression of rBMSCs was then examined. The CM derived from macrophages was found to increase the SPHK1 expression of rBMSCs under both normal and inflammatory conditions. Especially in inflammatory condition, the M1 macrophage derived-CM greatly increased the SPHK1 expression of rBMSCs. This indicated that the secreted factors derived from macrophages activated the SPHK1 activity of rBMSCs, therefore resulted in up-regulation of the S1P-S1PR1 signalling in rBMSCs; especially in the LPS-induced inflammatory condition.

To further confirm this finding, macrophages were co-cultured with rBMSCs using the trans-well system. The co-cultured cells were stimulated with or without LPS to mimic the normal or inflammatory conditions. Macrophages were found to induce the SPHK1 activity in the co-cultured rBMSCs, especially under LPS stimulation; which therefore resulted in enhanced S1P production and activated S1PR1 in rBMSCs. Therefore, it could be concluded that under the infection-induced inflammation, macrophages interacted with rBMSCs by producing secreted factors and inducing

SPHK1 activity in rBMSCs, which promoted S1P production. S1P acted in an autocrine manner to induce S1PR1 in rBMSCs, hence, the S1P-S1PR1 signalling was activated by the interaction between macrophages and rBMSCs.

As S1P was found to induce RANKL production, the RANKL expression of rBMSCs in the co-culture system was then examined. Same as the trend of S1PR1 activity, the mRNA and protein expression levels were found to be up-regulated in rBMSCs co-cultured with macrophages, especially under LPS-induced inflammation. It is yet to be confirmed which receptor that S1P acts on to induce RANKL. As demonstrated in Chapter 3, there was a correlation between S1PR1 and RANKL, which reflects on the interplay between rBMSCs and macrophages in the co-culture system. Therefore, it could be stated that the enhanced RANKL production is due to the activation of S1PR1. S1PR1 siRNA was then used to block S1PR1 in the co-culture system, and this S1PR1-silencing was found to result in reduced RANKL production in rBMSCs co-cultured with macrophages. This indicated that in rBMSCs, S1P-mediated RANKL induction was at least partially through S1PR1 activation. Hence, under infection-induced inflammation, the S1P-S1PR1 signalling, induced by the macrophage-BMSC interaction, acted as a key regulator in osteoclastogenesis by inducing RANKL production in BMSCs. Therefore, this is the first time that the S1P-S1PR1-RANKL axis is confirmed.

In the present study, up-regulated S1P-S1PR1 signalling in BMSCs was confirmed, which was due to the secreted factors derived from the co-cultured macrophages. The S1P-S1PR1 signalling was also found to be responsible for enhanced RANKL production in BMSCs. Under LPS-induced inflammatory condition, macrophages greatly activated the S1P-S1PR1 signalling in BMSCs, leading to an over-production of RANKL; hence, the interaction between macrophages and BMSCs played a significant role in osteoclastogenesis in infection-induced inflammatory disease, such as apical periodontitis. Moreover, the BMSC-derived S1P could also attract the migration of osteoclast-precursors (Ishii, et al., 2009), therefore greatly facilitating osteoclastogenesis. The macrophages also activated the S1P-S1PR1 signalling in BMSCs under normal condition. Although this up-regulation was comparably moderate, it suggests that the S1P-S1PR1-RANKL axis might be responsible for the physical bone remodelling. This study firstly proves that under the infection-induced inflammatory condition, the macrophages are activated and

polarized towards M1 phenotype while losing the ability of producing S1P. The interaction of macrophages and BMSCs activates SPHK1 of BMSCs, which eventually results in activation of the S1P-S1PR1 signalling and increase RANKL production. It is yet to be confirmed what kind of secreted factors derived from macrophages are responsible for activating SPHK1 in BMSCs. As TNF-α and IL-1 have been identified to induce SPHK1 activity (Pettus, et al., 2003; Xia, et al., 1998), it could be presumed that these pro-inflammatory cytokines take part in the activation of SPHK1 in BMSCs. One of the limitations of this study is that LPS could not fully simulate microbial infection; instead, it only partially mimic the infection-induced inflammation derived from Gram-negative microbes, such as the infection in apical periodontitis. In addition, as RAW cells are macrophage-like cells, it would be better to investigate if the similar results could be found in the primary human macrophages and BMSCs, which will be the future work of this study.

In conclusion, it is demonstrated that the interaction between macrophages and BMSCs is associated with the activation of S1P-S1PR1 signalling. Macrophages act as a regulator to trigger the SPHK1 activation of BMSCs, especially in infection-derived inflammatory condition. This interaction plays a crucial role by increasing the rate of osteoclastogenesis and bone loss, by activated S1PR1 inducing the expression of RANKL.

Chapter 5:

Suggested Statement of Contribution of Co-Authors for Chapter by Published Paper

In the case of this chapter

Title: **Macrophages induce osteogenesis under infection-induced inflammatory conditions in a S1P-S1PR1 signalling dependent manner**

Date, status, journal: March 2017, In preparation, Stem cell reports.

Contributor	Statement of contribution
Lan Xiao	Involved in the conception and design of the project, performed laboratory experiments, data analysis and interpretation. Wrote the manuscript.
Yinghong Zhou	Involved in the conception and design of the project, and reviewed the manuscript.
Rong Huang	Assisted with data analysis.
Shifeier Lu	Assisted with data analysis and sample preparation.
Yin Xiao	Involved in the conception and design of the project, and reviewed the manuscript.

Principal Supervisor Confirmation

I have sighted email or other correspondence from all Co-authors confirming their certifying authorship.

Name: Prof Yin Xiao Signature: 

Date: 3rd March 2017

Macrophages induce osteogenesis under infection-induced inflammatory conditions in a S1P-S1PR1 signalling dependent manner

5.1 ABSTRACT

Background: Abnormal osteogenesis has been observed in infection-induced inflammatory diseases, such as apical periodontitis, and the mechanism underlying this phenomenon remains unknown. Previous research has shown that S1PR1 can induce osteogenesis, therefore, the aim of this study is to investigate the role of S1P-S1PR1 signalling in the osteogenesis under condition of infection-induced inflammation.

Methods: *In vitro* co-culture system of macrophages and rBMSCs was applied with the stimulation of LPS. S1PR1-siRNA or a S1PR1-specific inhibitor was applied to block S1PR1 in rBMSCs co-cultured with macrophages. RT-qPCR, and western blot analysis were performed to detect the expression levels of osteogenic markers. Alizarin Red S staining was performed to evaluate the extent of osteogenesis.

Results: Following LPS stimulation the macrophages responded by significantly enhanced expression of osteogenic markers in the co-cultured rBMSCs, as well as increased mineral deposition. Blocking of S1PR1 reduced this enhancement, suggesting that S1P-S1PR1 signalling plays a significant role in induced osteogenesis in these conditions.

Conclusions: Under infection-induced inflammatory conditions, macrophages became stimulated and responded by interacting with BMSCs to activate the S1P-S1PR1 signalling pathway, which subsequently stimulated osteogenesis. S1P-S1PR1 signalling may, therefore, be responsible for the abnormal bone formation seen in infection-induced inflammatory diseases.

5.2 INTRODUCTION

There is ample evidence to show that the immune and skeletal systems are closely related and have many regulatory factors in common, such as cytokines, transcription factors, and signalling pathways (Takayanagi, 2007; Walsh, et al., 2006). A case in point is the immunomodulation of bone remodelling (Arron & Choi, 2000), a life-long process that consists of bone resorption (mediated by osteoclasts) and formation (mediated by osteoblasts) and a key function of bone metabolism (Hadjidakis & Androulakis, 2006; Raggatt & Partridge, 2010). Under normal physiological conditions, bone remodelling is a balanced process; the amount of resorption equals that of formation (Arron & Choi, 2000). However, during pathological conditions, such as inflammation, the balance between resorption and formation is disrupted by the inflammatory immune response, resulting in aberrantly stimulated bone resorption and a net bone loss, such as that seen in rheumatoid arthritis (RA) (Rodan & Martin, 2000), periodontitis (Taubman, et al., 2005), and apical periodontitis (Wang & Stashenko, 1993). Paradoxically, inflammation, which is generally considered as a destructive factor for bone (Redlich & Smolen, 2012), has been found to induce osteogenesis under certain conditions. Inflammation is necessary as an initial response in the healing injuries such as bone fractures (Mountziaris & Mikos, 2008). Also, excessive bone formation (such as osteophyte or bone spurs) has been found in chronical inflammations, such as arthritis (Oettmeier & Abendroth, 1989), and spondylarthrosis (Nathan, Pope, & Grobler, 1994). Similar phenomena are also seen in infection-induced inflammation, such as chronic osteomyelitis (Lew & Waldvogel, 2004) and apical periodontitis (Eliasson, Halvarsson, & Ljungheimer, 1984). However, the mechanism of this immune response-mediated bone formation has not been fully established.

Among the many and various types of immune cells, macrophages is the main cell type involved in the innate immune response (Mosser & Edwards, 2008) and is also considered a crucial regulator in osteogenesis. Macrophages encompass three main subpopulations of cells, consisting of non-activated M0 macrophages, pro-inflammatory M1 macrophages (induced by LPS or INF-γ), and immune-suppressive M2 macrophages (induced by IL-4 and IL-13) (Mantovani, et al., 2004). The M1 macrophages, in particular, have been shown to interact with BMSC–the precursor of osteoblast–via secreted factors and to induce the production of RANKL, a key factor

in osteoclastogenesis. Consequently, M1 macrophages are generally considered a negative factor in bone healing (Hashizume, et al., 2008; Kobayashi, et al., 2000; Lam, et al., 2000; Wei, et al., 2005; Zwerina, et al., 2007). However, recent studies have found that the M1 macrophages actually play a positive role in bone regeneration with macrophage infiltration during the early stage inflammation considered a critical factor in bone fracture repair. M1 macrophages have been shown to induce bone formation by producing OSM (Guihard, et al., 2012), and another M1 macrophage-derived factor, IL-6, also promotes bone formation (Bellido, et al., 1997; Blanchard, et al., 2009; Cho, et al., 2007; Itoh, et al., 2006; Sammons, et al., 2004). There is conflicting evidence as to the effects on osteogenesis of TNF-α and IL-1, both also derived from M1 macrophages (Hess, et al., 2009; Nanes, 2003; Perrien, et al., 2002), which suggests that the mechanisms of M1 macrophage-mediated bone formation is still far from fully resolved.

The bioactive lipid sphingosine-1-phosphate (S1P) has been identified to play a central role in immunoregulation by activating its five G-protein coupled receptors, termed sphingosine-1-phosphate receptor 1 (S1PR1)-S1PR5 (Rivera, et al., 2008). The S1P-S1PR1 signalling is a key modulator in the migration, differentiation and functionalized maturation of immune cells, which promotes inflammation by inducing the infiltration of activated immune cells (Rivera, et al., 2008; Spiegel & Milstien, 2011). S1P-S1PR1 signalling is involved in the interaction between cells from the monocyte/macrophage-lineage and osteoblast-lineage. This interaction is not only related with osteoclastogenesis by increasing RANKL production (Ryu, et al., 2006), but also osteogenesis under physical conditions by triggering the recruitment of osteoblast-precursors and enhancing the survival rate and mineralization of osteoblasts. Furthermore, the activation of MEK1/2-Erk1/2 and BMP-2/Smad signalling may be the tell-tale signs of osteogenesis induction (Pederson, et al., 2008; Sato, et al., 2012). Considering the complexity nature of the relationship between immune cells and bone-forming cells, the role of S1P-S1PR1 signalling in induced-osteogenesis during inflammation still warrants further investigation.

In the previous chapter, we demonstrated that macrophages interact with BMSCs to activation of S1P-S1PR1 signalling in BMSCs, especially under infection-induced inflammatory conditions. Since macrophages activated by inflammation have been found to induce bone formation and activated S1P-S1PR1 signalling can induce

osteogenesis, a testable hypothesis could be that the interaction between macrophages and BMSCs may affect osteogenesis *via* S1P-S1PR1 signalling under infection-derived inflammatory conditions. Hence, in this study we investigated the role of macrophages in infection-induced inflammatory bone formation, as well as its possible relation with the S1P-S1PR1 signalling.

5.3 MATERIALS AND METHODS

5.3.1 Cell culture

Rat BMSCs

The rat BMSCs (rBMSCs) were obtained from 8-10 week old Wistar male rats as described in previous study (Leboy, et al., 1991). Briefly, the rats were sacrificed and the back limbs were harvested. The femurs and tibias were dissected to remove all the skin and muscles, and then washed by PBS with 1% (v/v) penicillin/streptomycin (P/S; Gibco®, Life Technologies Pty Ltd., Australia) for three times. The dissected bones were transferred into a 10 cm petri dish containing Dulbecco's modified Eagle's medium (DMEM; Gibco®, Life Technologies Pty Ltd., Australia). The two ends of the bones were cut open and the bone marrow was flushed into a 50 mL tube by syringes filled with DMEM (containing 1% P/S). The obtained bone marrow was then washed once with PBS (containing 1% P/S) and resuspended in DMEM containing 10% (v/v) fetal bovine serum (FBS; In Vitro Technologies, Australia) and 1% P/S. The cell suspension was then transferred into a culture flask and cultured in incubators with 5% CO_2 at 37º C. After three days, the non-adherent cells were washed away by PBS, the adherent cells were then continually incubated in DMEM with 10% FBS and 1% P/S (culture medium was changed every 2-3 days). When reaching 80% confluence, the cells were passaged with 0.25% trypsin (containing 1mM EDTA, Gibco®, Life Technologies Pty Ltd., Australia) for 2 min. Cells within 5 passages were used for experiment. All experiment procedures were approved by the Ethics Committee of Queensland University of Technology.

RAW 264.7 cells

The murine-derived macrophage cell line, RAW 264.7 (RAW) cells were used to represent macrophage-like cells in this study. As a murine macrophage cell line, RAW cells have been proved to work reliably with BMSCs form other species (Chen, Wu, Gu, et al., 2014; Chen, Wu, Yuen, et al., 2014; Shi, et al., 2016). The RAW cells were cultured in DMEM with 10% FBS (heat-inactivated at 60 º C for over 30 min) and 1% P/S. The culture medium was changed every 2 to 3 days. After reaching 80% confluence, the cells were passaged by treating with 0.25% trypsin (containing 1mM EDTA) for 2 min.

5.3.2 *In vitro* cell co-culture

The rBMSCs were co-cultured with RAW cells under normal or LPS-induced inflammatory conditions (as seen in Fig 4.1B). A trans-well co-culture system was applied in this study. The cell culture inserts for 6-well plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) with 0.4 μm pore size were used to culture RAW cells. The rBMSCs were cultured in the companion plates (the ratio of numbers of rBMSCs to RAW cells is roughly 1:4). After 24 h of attachment, the inserts were then assembled onto the companion plates. Normal culture medium (supplemented with inactivated FBS) was used in the normal co-culture group. The RAW cells and rBMSCs cultured in normal culture medium served as normal-controls. In the LPS-induced inflammatory condition, co-cultured cells were stimulated with 100 ng/mL LPS (DMEM supplemented with 10% inactivated FBS and 1% P/S), the RAW cells and rBMSCs cultured in LPS-supplemented medium served as LPS-controls. After 12h of co-culture, rBMSCs were harvested for RNA and protein extraction.

5.3.3 S1PR1 siRNA Transfection

The S1PR1 siRNA ((5'GAC UAU GGC AAC UAU GAU A3', 5'UAU CAU AGU UGC CAU AGU C3', Product number: PDSIRNA2D, siRNA ID: SASI_Rn01_00101785, Sigma-Aldrich Pty. Ltd., Sydney, Australia) was used to block S1PR1 activity in this study (as seen in Fig 4.1B). The experiment was performed following the manufacturer's instructions. The rBMSCs for transfection were seeded in 6-well plates with culture medium (DMEM, 10% FBS, no P/S). After 24 h of cell attachment, forward transfection of S1PR1 siRNA was performed. 125 pmol S1PR1 siRNA was diluted in 250 ul Opti-MEM® I Reduced Serum Medium (Opti-MEM, Gibco®, Life Technologies Pty Ltd., Australia). Also, 4 μL Lipofectamine™ RNAiMAX (Life Technologies Pty Ltd., Australia) was diluted in the same amount of Opti-MEM. These two dilutions were gently mixed and incubated for 15 min at room temperature, and then transferred into the well. The cells were then incubated for 48 h in the incubator. After incubation, the rBMSCs were co-cultured with RAW cells using the trans-well system as described above. The universal negative control siRNA (MISSION® siRNA Universal Negative Control #1, Product number: SIC001, Sigma-Aldrich Pty Ltd., Sydney, Australia) was used in wells without S1PR1 siRNA.

5.3.4 Preparation of conditioned medium

Conditioned medium (CM) derived from RAW cells was obtained. Briefly, RAW cells were cultured in T175 flak to reach confluence, and then stimulated with 100 ng/mL LPS for 12 h. For co-culture under normal condition, cells were treated with normal culture medium for 12 h. After that, the medium was removed the cells were washed twice with PBS, and then incubated with serum-free DMEM for 12 h. The medium was collected and subjected to centrifugation (1000 g, 10 min, 4º C), then filtrated with a 0.2 μm filter (Millipore Corporation, Billerica, MA, USA) to remove cell debris. The filtered medium was stored at -80ºC for the further experiment.

5.3.5 Osteogenic differentiation of rBMSCs under normal / inflammation conditions

To investigate osteogenesis in the co-culture system under normal condition, the CM derived from RAW 264.7 cells (without LPS stimulation) was mixed with the osteogenic medium (DMEM, 20% FBS, 2% P/S, 20 mM β-glycerophosphate, 100 μM ascorbic acid and 200 nM dexamethasone; Sigma-Aldrich Pty Ltd., Sydney, Australia) in a ratio of 1:1. To investigate osteogenesis in co-culture system under LPS-induced inflammation (Fig 5.1), the CM derived from LPS-stimulated RAW 264.7 cells was mixed with osteogenic medium containing 200 ng/mL LPS (DMEM, 20% FBS, 2% P/S, 20 mM β-glycerophosphate, 100 μM ascorbic acid and 200 nM dexamethasone) in a ratio of 1:1. The mixed medium was then applied to culture rBMSCs. The rBMSCs cultured in normal osteogenic medium (DMEM, 10% FBS, 1% P/S, 10 mM β-glycerophosphate, 50 μM ascorbic acid and 100 nM dexamethasone) served as control for the normal co-culture, while the ones cultured in osteogenic medium (DMEM, 10% FBS, 1% P/S, 10 mM β-glycerophosphate, 50 μM ascorbic acid and 100 nM dexamethasone) supplemented with 100 ng/mL LPS served as control for the LPS-induced inflammatory co-culture. S1PR1 blockage was performed by application with the S1PR1-specific inhibitor, W146 hydrate (100 ng/ml, Sigma-Aldrich Pty Ltd., Sydney, Australia). The cells were stimulated for 1 day, and then harvested for RNA extraction or ALP activity assay. The cells stimulated for 10 days were harvested for Alizarin Red S staining and immunofluorescent staining.

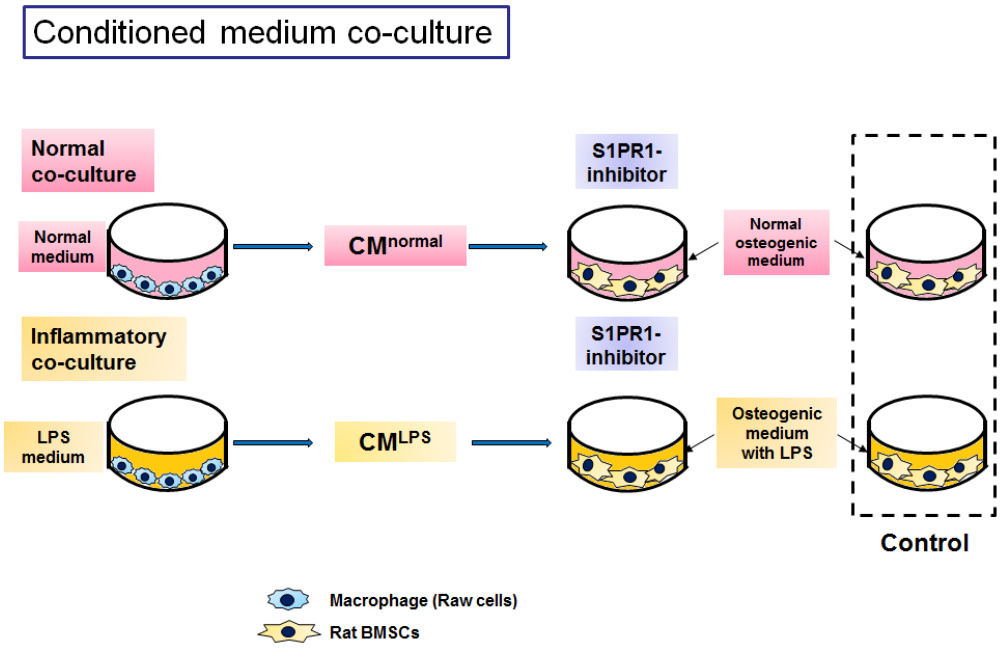

Figure 29. Osteogenic differentiation of rBMSCs co-cultured with macrophages (by conditioned medium) under normal / inflammation conditions. CM of macrophages under normal or LPS stimulation was collected and mixed with osteogenic medium or osteogenic medium with LPS; the mixed medium was then applied to rBMSCs. The rBMSCs applied with osteogenic medium or osteogenic medium with LPS were served as normal- or LPS-controls, respectively. S1PR1 was blocked by S1PR1-specific inhibitor, while vehicle was used in wells without S1PR1 siRNA. CM = conditioned medium.

5.3.6 RNA extraction, cDNA synthesis, and real time quantitative-PCR (RT-qPCR)

Total RNA was extracted from rBMSCs using the TRIzol Reagent (Ambion®, Life Technologies Pty Ltd., Australia). The cDNA was then synthesized from 1 µg total RNA by SensiFAST™ cDNA Synthesis Kit (Bioline (Aust) Pty Ltd., Australia) following the manufacturer's protocol. The real-time polymerase chain reaction (RT-qPCR) was carried out by the QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Australia) with SYBR® Green reagent (Applied Biosystems, Australia) according to the manufacturer's instructions. The RT-qPCR primers (Table 4.1) were designed based on cDNA sequences from the National Centre for Biotechnology Information (NCBI) sequence database and the primer specificity was confirmed by Primer-BLAST on the NCBI website. Analyses were performed on the following target genes: *S1PR1*, *ALP*, *COL-1*, *RUNX2* and *OCN*. The house keeping gene *18S* and *β-actin* were used as control. All reactions were run in triplicate for three independent experiments. Relative gene expression was normalized against *18S* or *β-actin* and calculated as previously described (Bookout & Mangelsdorf, 2003).

Table 5. Primer sequences for the gene (rat) investigated in this study

Gene name	Forward sequences	Reverse sequences
S1PR1	5'GTTGTCCGGGATTTGGTAGG3'	5'GATGCTCGTAGGGGTTAGAG3'
ALP	5'CCATTTCAGCCTCAGGATCG3'	5' TGGCCACGTTGGTGTTGAGT3'
COL-1	5'CCCCAAGGAGAAGAAGCATG3'	5'GAATCGACTGTTGCCTTCGC3'
RUNX2	5'TCTTTTGGGATCCGAGCACC3'	5'ATCTCCACCATGGTGCGGTT3'
OCN	5'GCCCTGACTGCATTCTGCCTCT3'	5'TCACCACCTTACTGCCCTCCTG3'
18S	5'CGGAACTGAGGCCATGATTAAG3'	5'GTATCTGATCGTCTTCGAACCTCC3'
β-actin	5'ATGCAGCCTGAAGAGGACTG	5'GGCTATGAAATCCAGGGCCT

5.3.7 Protein extraction and western blotting

Total protein was extracted from rBMSCs by the lysis buffer (20 mM HEPES (pH 7.4), 10% glycerol, 1% Triton X-100, 2mM EDTA) with the protease inhibitor cocktail (Roche Products Pty. Ltd., Dee Why, NSW, Australia). Measurements of protein concentration were performed by the BCA Protein Assay Kit (Thermo Fisher Scientific, VIC, Australia). For each sample, 10 μg of protein was loaded into a SDS-PAGE gel and then separated by running the gel. After that the protein was transferred to a nitrocellulose membrane (Merck Millipore, Billerica, USA). The membrane was blocked by the Odessy buffer (LI-COR Biosciences, Lincoln, USA) for 1 h at room temperature and then incubated with primary antibodies against ALP (1: 1000, Abcam, Cambridge, UK) and COL-1 (1:3000, Invitrogen Pty Ltd., Australia); α-Tubulin (1: 5000, Abcam, Cambridge, UK) was used as a loading control. After incubated at 4℃ overnight, the membrane was washed and then incubated with Anti-rabbit IgG IRDye 800 conjugated secondary antibody (1: 10000, Rockland, Gilbertsville, Pennsylvania, USA) for 1 h at room temperature. After three-times of washing, the membranes were scanned by Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, USA) according to the manufacturer's instructions. Quantification of band intensities was obtained by ImageJ software. All experiments were replicated by three times.

5.3.8 ALP activity assay

The ALP activity of rBMSCs was tested by Alkaline Phosphatase Assay kit (Colorimetric) (Abcam, Cambridge, UK) according to the manufacture's instruction. The rBMSCs were harvested by the ALP Assay Buffer. The protein concentration was measured by the BCA Protein Assay Kit (Thermo Fisher Scientific, VIC, Australia). The cell samples were diluted by the Assay Buffer in a ratio of 1:3, and then transferred to a 96-well plate. The pNPP solution was added to the sample wells. ALP Enzyme Solution was added to the pNPP standard wells. The plate was then incubated at 25℃ for 60 min in the dark. After incubation, reactions were stopped by the Stop Solution. The measurement was performed immediately by a microplate reader (SpectraMax, Plus 384, Molecular Devices, Inc., USA) at OD 405 nm. For each sample, the test was performed in triplicate, and the ALP activities were calculated according to the manufacture's instruction. All the results were expressed as the calculated ALP activity / protein concentration.

5.3.9 Alizarin Red S staining

After 10 days of osteogenic differentiation, the rBMSCs were washed twice with PBS and fixed with 4% PFA for 20 min at room temperature. After that, the cells were rinsed with distilled water, and then stained in the 1% Alizarin Red S (Sigma-Aldrich Pty Ltd., Sydney, Australia) solution (pH: 4.1-4.3) for 20 min at room temperature. After washing with distilled water, the samples were air-dried and observed under the microscope (Eclipse TS100, Nikon Australia Pty Ltd.).

5.3.10 Statistical analysis

All data were subjected to statistical analysis using one-way ANOVA, followed by the Student-Newman-Keul test at a = 0.05. Pearson correlation was used for correlation analysis on numbers of $S1PR1^+$ cells with osteoclasts and $RANKL^+$ cells (a = 0.05). A $p< 0.05$ was considered to significantly different. Data were analysed by SPSS 13.0 (SPSS Inc., Chicago, IL). All data were presented as mean ± standard deviation (SD).

5.4 RESULTS

5.4.1 Macrophages induced the osteogenic differentiation of rBMSCs

A trans-well co-culture system was used to determine the impact of macrophages on rBMSCs under normal or infection-induced inflammatory conditions and LPS stimulation used to simulate infection-mediated inflammation. After 12 h of co-culture under inflammatory conditions (Fig 30), we found significantly increased mRNA levels of the osteogenic markers, *ALP*, *COL-1*, *RUNX2*, and *OCN* in rBMSCs co-cultured with macrophages, whereas in the LPS negative (normal) co-cultures only *COL1* showed significantly increased expression. The western blot results (Fig 31) also showed that early stage osteogenic markers were induced in rBMSCs co-cultured with macrophages under both conditions. These results suggested that in conditions mimicking infection induced inflammation, macrophages induced osteogenic differentiation and gene expression.

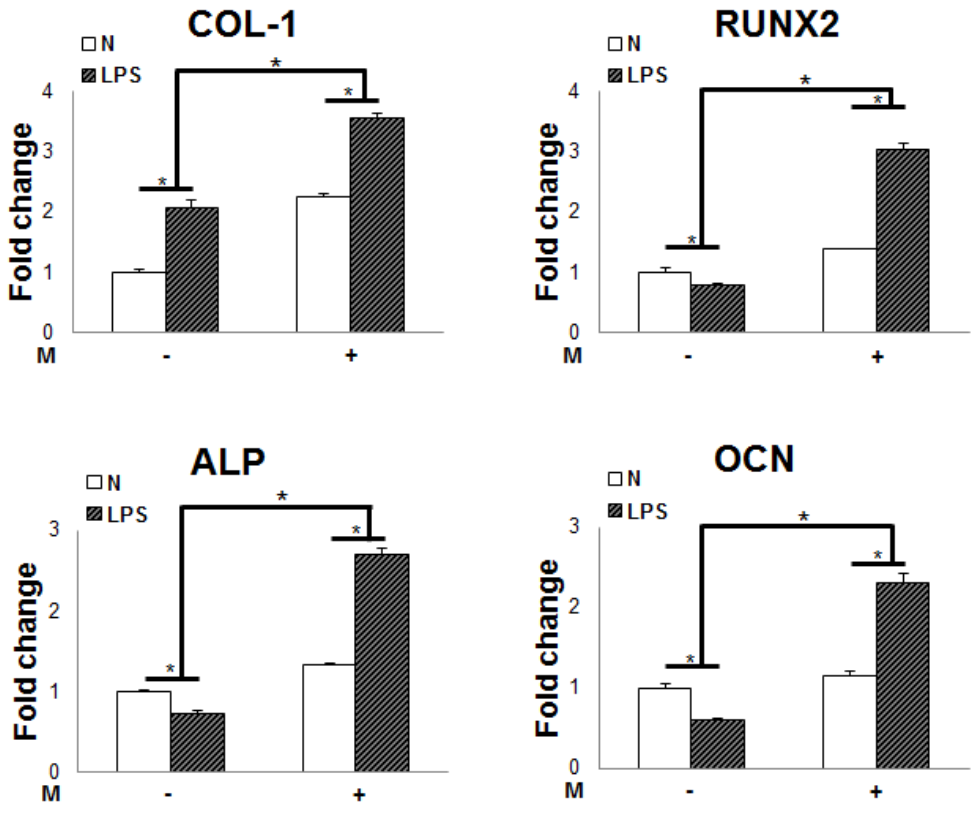

Figure 30. Up-regulated osteogenic markers in rBMSCs co-cultured with macrophages (n=3). Under both normal and LPS-stimulation conditions, the mRNA levels of osteogenic markers (*COL-1*, *RUNX2*, *ALP* and *OCN*) in rBMSCs were significantly up-regulated when co-cultured with macrophages (*p<0.05). M = co-cultured with macrophages, N = normal medium, LPS = medium with LPS.

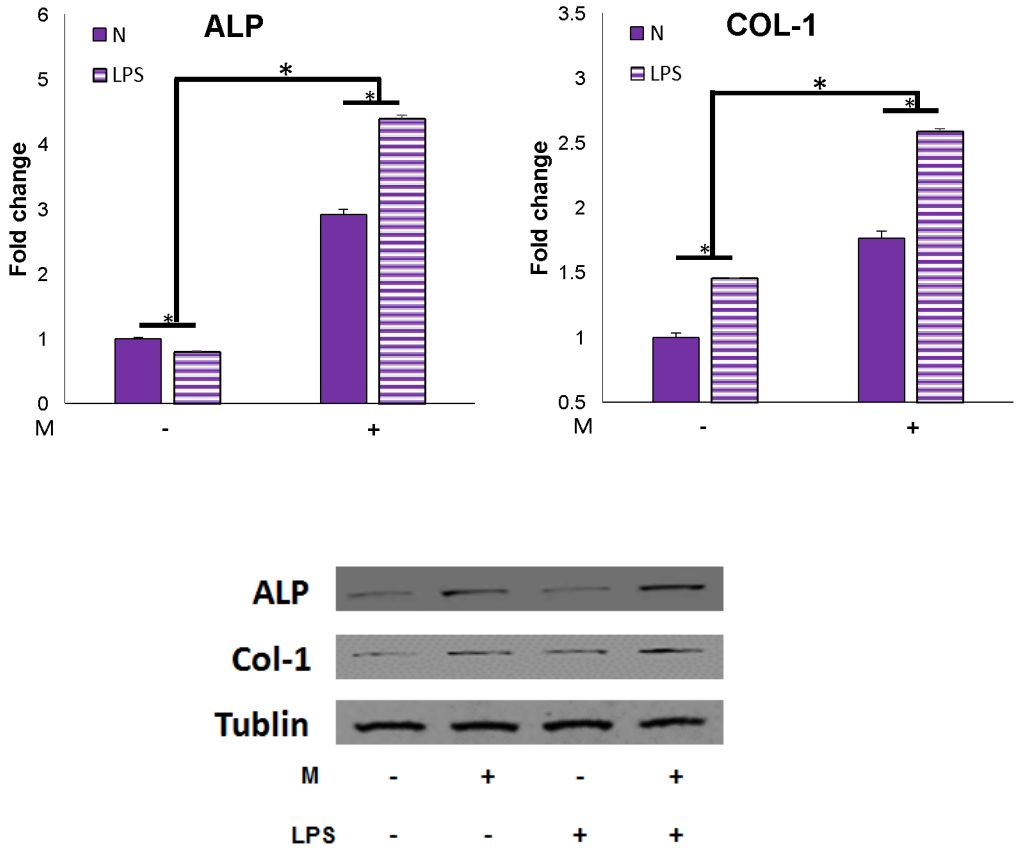

Figure 31. Up-regulated osteogenic markers in rBMSCs co-cultured with macrophages (n=3). Under both normal and LPS-stimulation conditions, the protein levels of early stage osteogenic markers (COL-1 and ALP) in rBMSCs were significantly up-regulated when co-cultured with macrophages (*p<0.05), indicating that macrophages induced the differentiation and activation of osteoblasts, especially under the LPS-induced inflammatory conditions. M = co-cultured with macrophages, N = normal medium, LPS = medium with LPS.

5.4.2 The macrophage-induced osteoblast differentiation was due to the activation of S1P-S1PR1 signalling

S1PR1 was blocked by the S1PR1-siRNA in order to assess the role of S1P-S1PR1 signalling in the activation of osteoblasts. S1PR1-siRNA treatment significantly reduced the mRNA expression levels of osteogenic markers (*COL-1*, *RUNX2*, *ALP* and *OCN*) in the rBMSCs co-cultured with macrophages (Fig 32). The rBMSCs also showed decreased protein expression levels of the early stage osteogenic markers (Fig 33). Hence, the S1P-S1PR1 signalling played an essential role in the activation of osteoblasts in the inflammatory co-culture.

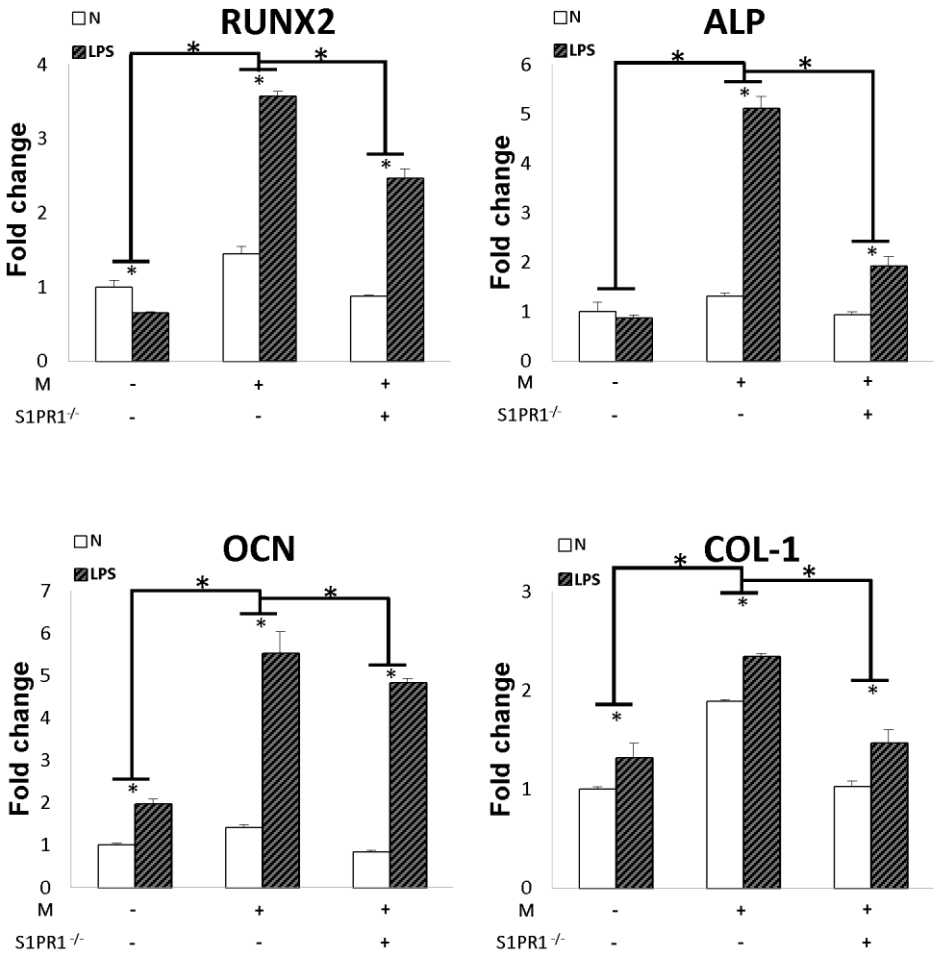

Figure 32. The impacts of S1PR1 regulation on osteogenic markers expression (n=3). Under LPS-stimulation conditions, the mRNA levels of osteogenic markers (*RUNX2*, *ALP*, *COL-1*, and *OCN*) in rBMSCs co-cultured with macrophages were significantly down-regulated when S1PR1 was inhibited (*p<0.05). M = co-cultured with macrophages, N = normal medium, LPS = medium with LPS, S1PR1 -/- = S1PR1 siRNA.

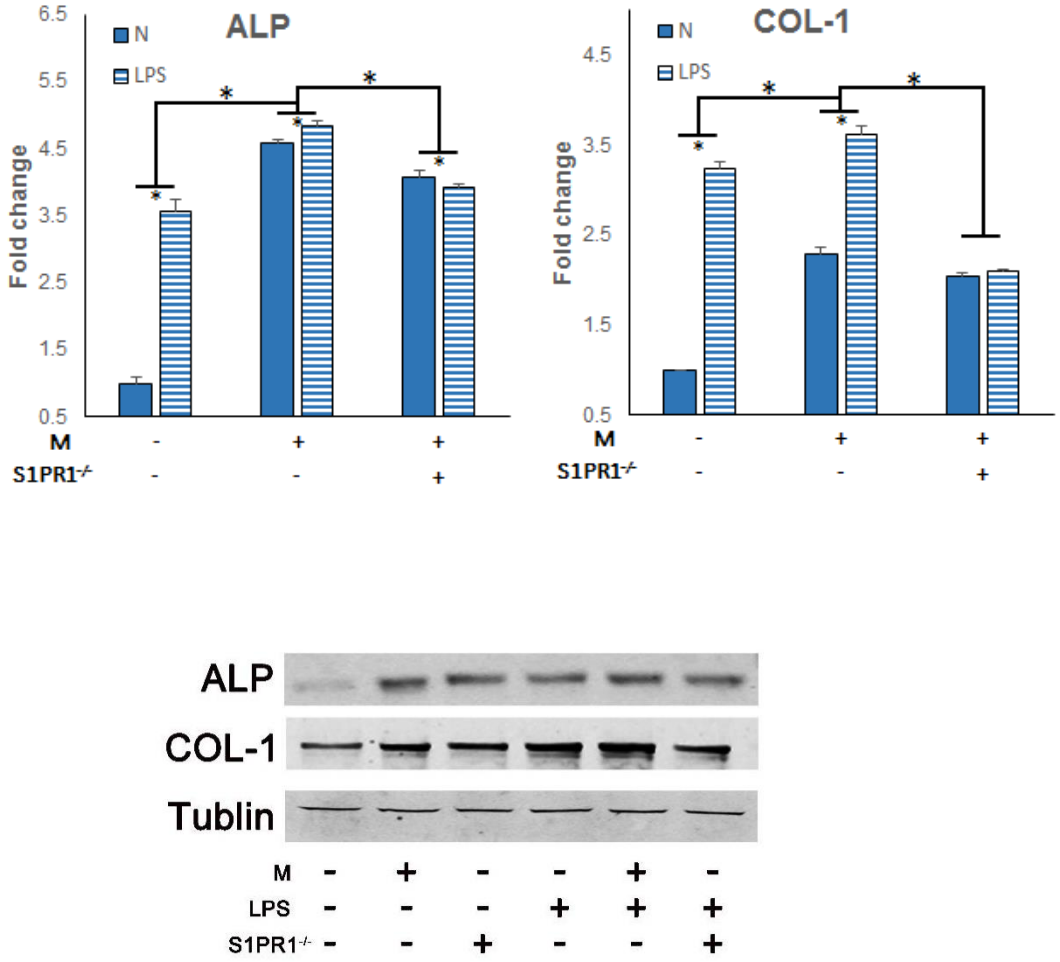

Figure 33. The impacts of S1PR1 regulation on osteogenic markers expression (n=3). Under both normal and LPS-stimulation conditions, the protein levels of early stage osteogenic markers (COL-1 and ALP) in rBMSCs co-cultured with macrophages were significantly down-regulated when applied S1PR1 was inhibited (*p<0.05), suggesting that the activation of osteoblasts in the inflammatory co-culture was due to the S1P-S1PR1 signalling. M = co-cultured with macrophages, N = normal medium, LPS = medium with LPS, S1PR1 -/- = S1PR1 siRNA.

5.4.3 Macrophages induced osteogenesis through activation of the S1P-S1PR1 signalling

To confirm whether macrophages induce osteogenesis under LPS-induced inflammation, and whether the S1P-S1PR1 signalling plays a role in the induced osteogenesis, we tested the effect of macrophages on osteogenesis under normal and LPS-induced inflammatory conditions. Macrophage CM was used to stimulate the rBMSCs. CM derived from LPS-stimulated macrophages was applied to rBMSCs in osteogenic medium with the supplementation of LPS (inflammatory co-culture), whereas CM derived from un-stimulated macrophages was applied to rBMSCs in normal osteogenic medium (normal co-culture). The S1PR1 specific antagonist W146 hydrate was used to block S1PR1. As shown in Fig 34a to f, CM derived from macrophages significantly enhanced the mRNA levels of the osteogenic markers, as well as ALP activity under both normal and inflammatory conditions. Furthermore, when S1PR1 was inhibited, the enhanced expressions of the osteogenic markers and ALP activity were reduced. The Alizarin Red S staining was performed to identify mineralization nodules. As shown in Fig 34g, after ten days of osteogenic differentiation, the macrophages CM had a significant inductive effect on mineralization of rBMSCs under both conditions, which was reduced by the S1PR1 antagonist. Thus, macrophages induced osteogenesis under both normal and LPS-induced inflammatory conditions, which was achieved by activating the S1P-S1PR1 signalling.

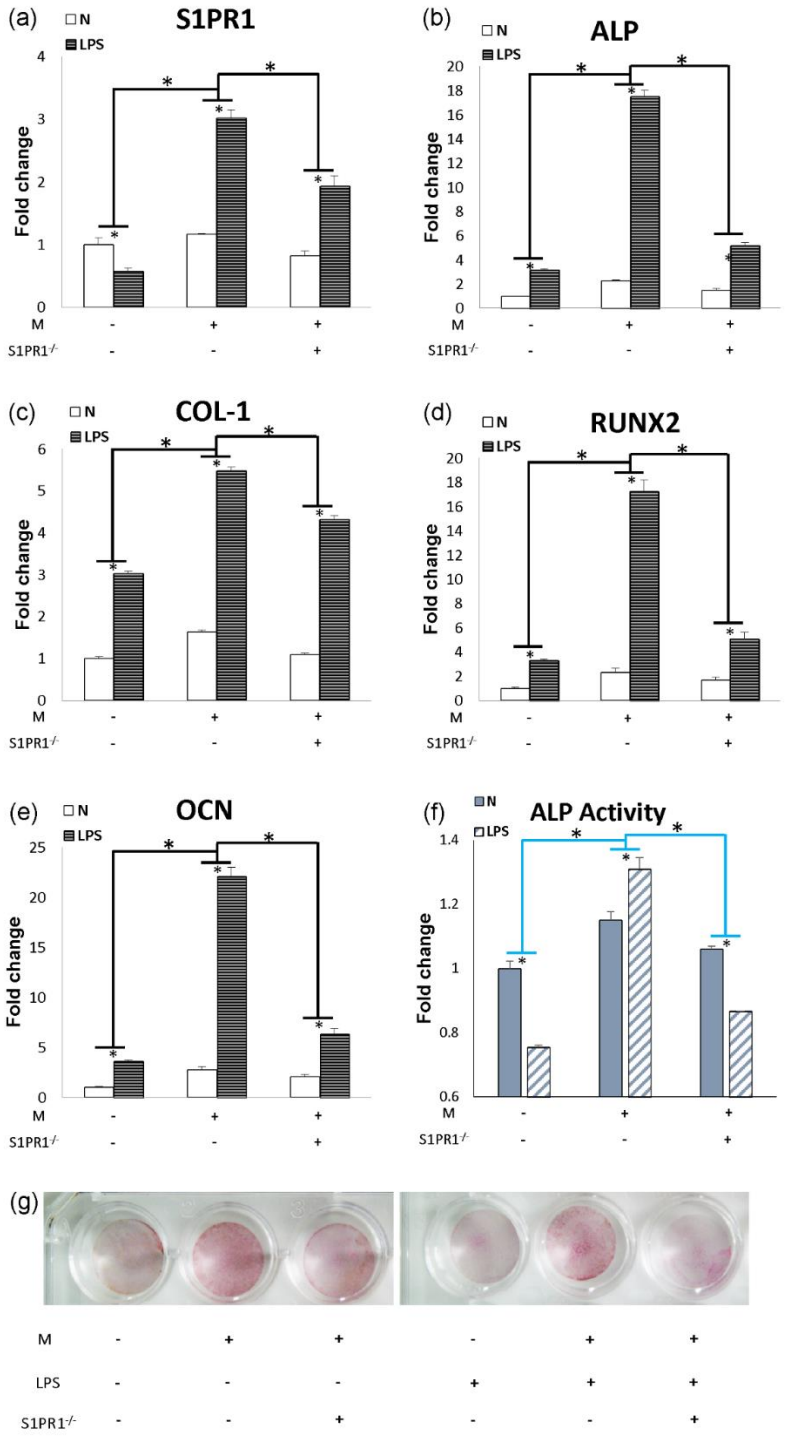

Figure 34. The role of S1P-S1PR1 signalling in macrophage-induced osteogenesis (n=3). (a) – (e): Under both normal and LPS-stimulation conditions, the mRNA levels of osteogenic markers (RUNX2, ALP, COL-1, and OCN) in rBMSCs were significantly up-regulated when co-cultured with macrophages (by CM). This up-regulation was significantly down-regulated when S1PR1 was blocked (*p<0.05). (f): ALP activity in rBMSCs co-cultured with macrophages was significantly enhanced under both normal and LPS-stimulation conditions; this enhancement was significantly reduced after S1PR1 blockage. (g): Induced mineralization could be found in rBMSCs co-cultured with macrophages (by CM) under both conditions; which was reduced by S1PR1 blockage. All these data suggested that macrophages induced osteogenesis under LPS- induced inflammatory conditions in a S1P-S1PR1 signalling dependent manner. M = co-cultured with macrophages (by stimulation with CM derived from macrophages), N = normal medium, LPS = medium with LPS, S1PR1 -/- = S1PR1 inhibitor.

5.5 DISCUSSION

Inflammation has long been recognised as being partially responsible for bone destruction. The evidence for this is that activated immune response under inflammation has been shown to trigger RANKL-RANK signalling directed osteoclastogenesis (Arron & Choi, 2000), resulting in the enhanced bone resorption and bone loss that is seen in inflammatory bone destructive diseases such as rheumatoid arthritis (RA) (Rodan & Martin, 2000), periodontitis (Taubman, et al., 2005), and apical periodontitis (Wang & Stashenko, 1993). However, in some cases, inflammation may also result in aberrantly-induced bone formation. A typical case is osteophyte formation – abnormally formed bone tissue found around damaged joints such as arthritic tissues (Oettmeier & Abendroth, 1989). The similar phenomenon also happens in infection-induced bone destructive diseases such as osteomyelitis (Lew & Waldvogel, 2004) and apical condensing osteitis (Eliasson, et al., 1984) – a special subset of apical periodontitis, which features with over-induced bone formation. Compared with osteoclastogenesis, the role and mechanisms of inflammation in osteogenesis, especially under the condition of infection, are poorly understood.

Recent studies have found that macrophages – the major effector cells in the innate immune response that are generally considered to induce osteoclastogenesis – also play a key role in inducing osteogenesis (Wei, et al., 2005; Zwerina, et al., 2007). The main macrophage phenotype in inflammation, the pro-inflammatory M1 type, have been found to interact with osteoblast-precursors (known as BMSCs) and induce osteogenesis by the secreted factor OSM (Guihard, et al., 2012). Furthermore, another M1 macrophage-derived factor, IL-6, is also found to promote osteogenesis (Bellido, et al., 1997; Blanchard, et al., 2009; Cho, et al., 2007; Itoh, et al., 2006; Sammons, et al., 2004). As M1 macrophage differentiation and activation have been identified in infection-induced disease (Mantovani, et al., 2004), it could be presumed that the M1 macrophages might interact with BMSCs and play a role in the infection-induced inflammatory osteogenesis. In Chapter 4, we found that the interaction between macrophages and rBMSCs resulted in activation of S1P-S1PR1 signalling. Some studies have found that this signalling pathway is associated with osteogenesis, which prompted the hypothesis that the S1P-S1PR1 signalling should affect osteogenesis under this infection-induced inflammatory condition (Pederson, et al., 2008). Therefore, this study aimed to investigate the effect of macrophages in osteogenesis

under infection-induced inflammation, and the role of S1P-S1PR1 signalling in this process.

To investigate the impact of macrophages in the infection-induced inflammatory osteogenesis, the trans-well co-culture system of macrophages and rBMSCs was stimulated with or without the supplementation of LPS to mimic the interacted macrophages and rBMSCs under normal or infection-induced inflammatory conditions. The expressions of osteogenic markers were examined in the co-cultured rBMSCs. Increased mRNA levels were found in the rBMSCs co-cultured with macrophages, which was more obvious in the LPS co-culture. At the same time, protein levels of the early stage markers in these co-cultured rBMSCs were also observed. These results indicated that the rBMSCs were induced by the co-cultured macrophages to initiate differentiation towards osteoblasts. This induction was more obvious in the LPS co-culture, suggesting macrophages greatly induce the osteoblast phenotype in the infection-induced inflammation.

To determine if the activated osteoblast differentiation was due to the up-regulated S1P-S1PR1 signalling (as was demonstrated in Chapter 4), the effect of S1PR1-inhibition on the expression of osteogenic markers was then investigated. S1PR1 silencing led to a significant downregulation of mRNA and protein expression of osteogenic markers in rBMSCs co-cultured with macrophages in the infection-induced inflammatory conditions, indicating the S1PR1 could be a key factor in the macrophage-induced osteoblast activation; therefore, the macrophage-induced osteoblast differentiation was partially achieved through the activated S1P-S1PR1 signalling in rBMSCs.

To assess whether macrophages could induce osteogenesis through the S1P-S1PR1 signalling pathway, a similar macrophage-BMSC co-culture system was established to simulate the osteogenic differentiation. To avoid the possible impact of the osteogenic medium on macrophages, an in-direct co-culture system using macrophage CM was applied. The rBMSCs were stimulated with M1 macrophage-derived CM and then supplemented with LPS and osteogenic medium to mimic the situation of LPS co-culture (in the transwell system); while the normal co-culture (in the transwell system) was simulated by culturing rBMSCs with CM of M0 macrophages mixed with normal osteogenic medium. The S1PR1 specific antagonist W146 hydrate was used to block S1PR1 instead of S1PR1 siRNA, since the RNA

silencing effect could not last for 10 days. Similar to the findings in trans-well co-culture system, the rBMSCs in the inflammatory conditions resulted in the up-regulation of osteogenic markers when stimulated with the M1 macrophage-derived CM; however, this effect was reduced following S1PR1 inhibition with W146. CM derived from M0 macrophages also resulted in slightly increased expression of osteogenic markers in rBMSCs and this effect was also reduced by S1PR1 inhibition. Accordingly, rBMSCs cultured in macrophage CM showed enhanced ALP activity under both normal and inflammatory conditions. After ten days of osteogenic differentiation, the Alizarin Red S staining ultimately showed that the macrophage CM enhanced the osteogenesis under both normal and infection-induced inflammatory conditions, which was also reduced by S1PR1 inhibition. Therefore, these results are firm evidence that macrophages induce osteogenesis by up-regulation of the S1P-S1PR1 signalling in rBMSCs under infection-induced inflammatory conditions.

An intriguing finding is the observation that the pro-inflammatory M1 macrophages resulted in a more robust osteogenic response than did the M2 macrophages, the phenotype which is considered more beneficial for tissue regeneration (Guihard, et al., 2012). A possible explanation for this is that during the early stages of bone healing, which is characterized by an acute inflammatory phase, the M1 macrophages act as a key factor in the differentiation of osteoblasts, whereas M2 macrophages take part in the later stage of osteogenesis by inducing the maturation and terminal mineralization of osteoblasts. The transition from the M1 to M2 phenotype is, therefore, considered to be crucial aspect of bone healing and *de novo* bone formation (Loi, et al., 2016). In the preceding chapter, it was found that the macrophages were capable of inducing the SPHK1 activity in the co-cultured BMSCs, thereby promoting the S1P production. S1P has been identified to play a regulatory role in macrophage polarization, which induces the transition of M1 to M2 phenotype by activating S1PR1 in macrophages (Hughes, et al., 2008). It is, therefore, likely that during the process of bone healing, the early stage inflammation induces the activation of M1 macrophages, which signals via the SHPK1-S1P-S1PR1 axis in BMSCs, thereby initiating osteogenesis. It is also possible that secreted S1P could act on the macrophages and lead to the gradual conversion of M1 to M2 phenotype, thereby creating suitable conditions for later stage osteogenesis. Further research is required to test this hypothesis. Also, the inflammatory osteogenesis induced by M1 macrophages

may result in abnormal bone formation, as exemplified by the dysfunctional *de novo* bone found in osteomyelitis (Lew & Waldvogel, 2004). Hence, further research on the chemical constitution and structure of bone tissues formed under infectious inflammation should be performed.

Taken together, it is demonstrated that LPS-stimulated macrophages are capable of inducing osteogenesis in infection-induced inflammatory conditions, by activating the S1P-S1PR1 signalling in BMSCs *in vitro*. These findings go some way towards explaining a molecular mechanism for the bone formation seen in infection-related inflammatory diseases.

Chapter 6: Conclusions

6.1 SUMMARY

Skeletal bones undergo a lifelong process termed bone remodelling, which consists of osteoclasts-driven bone resorption and osteoblasts-driven bone formation (Raggatt & Partridge, 2010). Bone remodelling is highly regulated by immune modulators to keep the balance between bone resorption and formation. This is a fundamental interaction between the immune and skeletal systems and describes a physiological process known as osteoimmunology (Arron & Choi, 2000). The immune response under pathological conditions, such as inflammation, results in an imbalance between bone resorption and formation, which eventually leads to bone diseases, as seen in rheumatoid arthritis (RA) (Rodan & Martin, 2000), chronic osteomyelitis (Lew & Waldvogel, 2004), periodontitis (Taubman, et al., 2005), apical periodontitis (Wang & Stashenko, 1993). A better understanding of how the immune system modulates bone remodelling under the inflammatory conditions will help us develop better treatments to target inflammatory bone-destructive diseases.

The immune and skeletal systems are integrated through number of factors. Key among these factors is the multifunctional sphingolipid metabolite-S1P which, not only acts as a key factor in immune-regulation, but also directly regulates the process of bone remodelling and as such plays an essential role in osteoimmunology. The function of S1P is achieved by binding with cognate receptors, the S1PRs, of which five separate ones have been identified (Spiegel & Milstien, 2011). S1PR1, which is widely expressed in the immune and skeletal system (Aarthi, et al., 2011), is found to be greatly involved in the S1P-derived regulation in osteoimmunology (Rivera, et al., 2008). The role of S1P-S1PR1 signalling in osteoimmunology has intrigued researchers since, besides being capable of inducing inflammatory response and therefore facilitating osteoclastogenesis, it also acts as a positive factor in osteogenesis. This makes its role in bone remodelling far from straight forward, especially under pathological conditions such as inflammation.

Within the local-inflammatory tissues of bone-destructive disease, such as RA, increased S1P concentration and enhanced S1PR1 expression has been identified. It has also been shown that modulating S1P-S1PR1 signalling can have therapeutic

effects on bone loss (Maceyka, et al., 2012). However, the role of this signalling in the infection-induced bone loss (e.g. apical periodontitis, osteomyelitis) has not been well studied. Compared with non-infectious inflammation, the status of S1P-S1PR1 signalling in infection-induced inflammation may be different, since the invading pathogens affect the tissues in a number of ways. Hence, the role of S1P-S1PR1 signalling in the infection-induced bone loss is a subject worthy of research. The aim of this project was to reveal the functions and mechanisms of S1P-S1PR1 signalling in infection-induced inflammatory bone remodelling.

The study chose to focus on apical periodontitis–one of the most common oral diseases caused by infection–as the model condition in which to study the role of S1P-S1PR1 signalling in pathogenic bone loss. In *Chapter 3* we report a study in which we examined and compared the expression levels of S1P-S1PR1 signalling in periapical lesion tissues and normal periapical tissues isolated from human subjects. Up-regulation of S1P-S1PR1 signalling was identified in the lesion tissues, which was accompanied by up-regulated expression of RANKL (the key factor in osteoclastogenesis) in the same tissues. Double staining of S1PR1 and RANKL support the notion of a possible connection between the S1P-S1PR1 signalling and RANKL, which precipitate the bone-destruction seen in apical periodontitis. To further investigate this connection, an induced rat periapical lesion model was applied to study the relationship between S1P-S1PR1 signalling, RANKL, and osteoclasts. The results from this study indicated that there were positive correlations among these three factors and that the up-regulated S1P-S1PR1 signalling might result in increased RANKL expression and subsequent osteoclastogenesis. To test this hypothesis, the S1P-S1PR1 signalling was modulated by FTY720 (an inhibitory S1P analogue) in an induced periapical lesion models in rats. This approach strongly reduced RANKL expression and was associated with a significant reduction of osteoclastogenesis and bone destruction in the apical periodontitis model. Hence, in this report, it is identified that aberrant up-regulation of S1P-S1PR1 signalling leads to the bone loss associated with infection-induced apical periodontitis, and the results support the view that suppression of RANKL reduces osteoclastogenesis and, consequently, bone loss.

Although S1P-S1PR1 signalling activation is known to be associated with apical periodontitis, it is yet unknown what activates this signalling. Macrophages, which are activated and played a central role in the innate immune response against pathogen

invasion, not only act as precursors for osteoclasts, but also interact with pre-osteoblasts (known as BMSCs) to regulate the RANKL production and hence modulate osteoclastogenesis (Takeshita, et al., 2000). Also, since cells from the macrophage-monocyte lineage have been identified as important sources of S1P and, further, that S1PR1 is expressed by macrophages and BMSCs, it was hypothesised that S1P-S1PR1 signalling was involved in the macrophage-BMSC interaction. In *Chapter 4*, this hypothesis was tested using a macrophage-BMSC co-culture system that was stimulated with LPS in order to mimic a bacterial infection. S1P expression of macrophages fell significantly following LPS stimulation; however, other secreted factors from the macrophage significantly up-regulated the SPHK1 activity of BMSCs and, therefore, resulted in increased S1P production of BMSCs which were likely to activate S1PR1 in an autocrine manner. Therefore, under infection-induced inflammatory conditions, activated macrophages interacted with BMSCs, which resulted in up-regulated S1P expression in BMSCs and ultimately an autocrine S1PR1 activation.

It was also observed that RANKL expression was induced in BMSCs with activated S1PR1. Although S1P has been found to induce RANKL expression in pre-osteoblasts, it was still unknown which particular receptor (e.g. S1PR1, S1PR2 and S1PR3) was required for this effect. To test whether S1PR1 was necessary for in S1P-induced RANKL production, S1PR1 was down-regulated in BMSCs in the macrophage-BMSC co-culture and showed a significant reduction of RANKL expression associated with the S1PR1 down-regulation. This is strong evidence for an essential role of S1PR1 in S1P-induced RANKL expression. Taken together, in Chapter 4, it is found that macrophages, in response to LPS stimulation, interacted with BMSCs to induce the S1P production. Subsequently, S1P activated S1PR1 in BMSCs which induced the RANKL expression, and ultimately promoted osteoclastogenesis.

Since bone remodelling is the net result of bone resorption versus bone formation, it is necessary to know how the S1P-S1PR1 signalling affects osteogenesis. In *Chapter 5*, canonical osteogenic markers were found to be up-regulated in BMSCs co-cultured with macrophages (especially when stimulated with LPS). Osteogenic marker expression, ALP activity, and calcification nodule formation all decreased following S1PR1 blockage, which suggest a major role for S1P-S1PR1 signalling in suppressing

osteogenesis in response to infection-induced inflammatory conditions. Therefore, under infection-induced inflammation, the interaction between macrophages and BMSCs leads to the activation of S1P-S1PR1 signalling in BMSCs, which then induce osteogenesis. Although inflammation is generally thought to lead to bone loss (Redlich & Smolen, 2012), it has also been shown to induce abnormal osteogenesis under chronical inflammations, as exemplified by bone spurs in arthritis (Oettmeier & Abendroth, 1989) and spondylarthrosis (Nathan, et al., 1994). Excessive bone formation has also been found in infection-induced inflammation, such as chronic osteomyelitis (Lew & Waldvogel, 2004) and apical periodontitis (Eliasson, et al., 1984). However, the mechanism behind this inflammatory bone formation is not fully established. Hence, this is the first study of its kind to elucidate the role of S1P-S1PR1 signalling in the interaction between the immune and skeletal systems that results in abnormal osteogenesis under infection-induced inflammation, and provides new insights into osteoimmunology.

6.2 LIMITATIONS AND FUTURE DIRECTIONS

In this thesis, the role of S1P-S1PR1 signalling in macrophage-BMSC interaction was mainly studied *in vitro*. One can only draw a limited number of conclusions from such data and the logical next step will be to verify these results *in vivo*, ideally in genetically modified mouse models, such as a conditional S1PR1-knock down in osteoblast lineage cells. Also, in *Chapter 3*, the S1P-S1PR1 signalling modulation *in vivo* was achieved by the application of S1P analogue. Only *in vitro* data has been confirmed in *Chapter 4* that S1P-derived RANKL production was due to the expression of S1PR1. It would be better if we could use S1PR1-specific inhibitor to further confirm its role in osteoclastogenesis *in vivo*.

In *Chapter 4*, we found macrophage-derived factors were induced by activated SPHK1 activity in the co-cultured BMSCs. However, it is still unknown what factors are responsible for this activation. This will be confirmed in the future research. Still, in Chapter 4, the relation of S1PR1 and RANKL was verified by S1PR1 regulation. To ultimately prove the S1PR1 is correlated with RANKL expression, the Co-immunoprecipitation will be performed in our future study.

In *Chapter 5* of this project, the main focus was on how S1P-S1PR1 signalling affected BMSCs under the conditions mimicking infection-induced inflammation. However, as there is strong evidence that S1P takes part in macrophage polarization, it could be hypothesised that the BMSCs-derived S1P may in turn affect the phenotype of macrophages in the co-culture. Further research will be performed to establish if this is the case. Also, all the in vitro experiments in *Chapter 4* and *5* were done on rat primary BMSCs and the RAW 264.7 macrophage cell line. Ideally it would be better to explore the role of S1P-S1PR1 signalling in macrophage-BMSC interaction and bone remodelling using human primary cells, and this will be done in future work. Moreover, although enhanced osteogenesis has been found in infection-induced inflammation, it is still unknown whether or not the structure and components of this bone is equal to that of bone formed under normal conditions. Future work will be performed to compare the chemical components, structure and mechanics of the bone formed under physical and infectious inflammatory conditions.

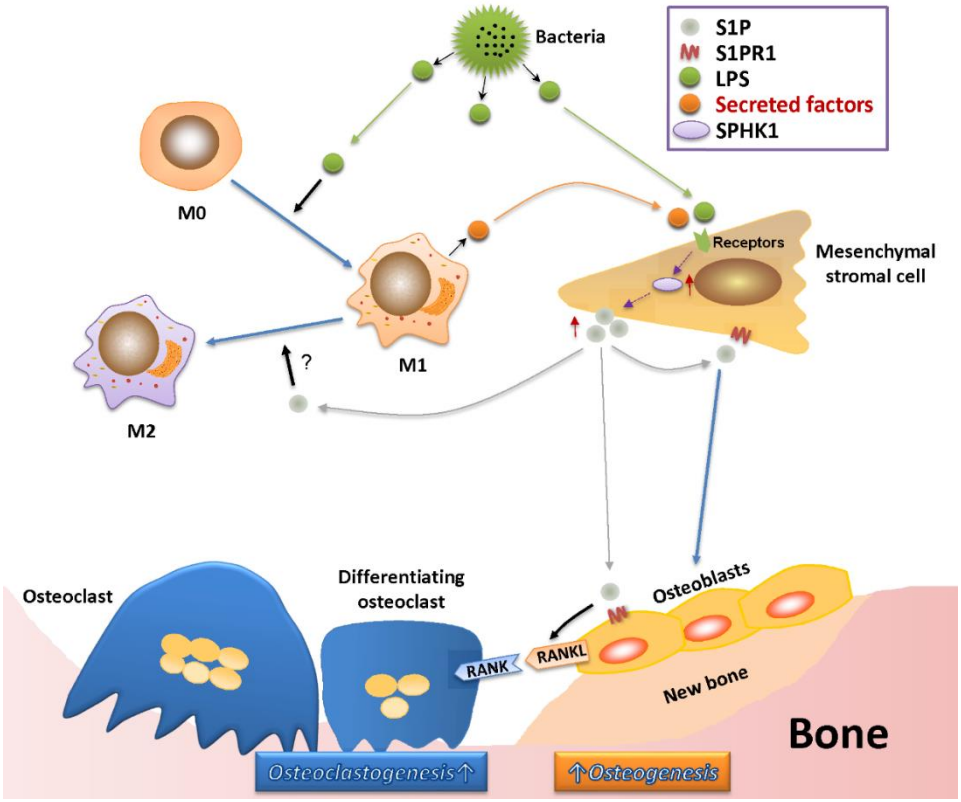

Figure 35. Summary of the whole study. In the infection-induced inflammation, the components of the invading bacteria (i.e.: LPS) activates macrophages and induces the M1 phenotype. The secreted factors from M1 macrophages then induce SPHK1 activity in BMSCs and therefore results in over-production of S1P. S1P acts its receptor S1PR1 in BMSCs in an autocrine manner. The activated S1P-S1PR1 signalling results in multiple affections on bone remodelling. On one hand, it induces the osteoblast differentiation and osteogenesis; on the other hand, it enhances the RANKL production of the osteoblast-lineage cells, which eventually induces osteoclastogenesis. Moreover, the over-produced S1P might affect the phenotype of macrophages by inducing a shift from the M1 to M2 phenotype.

A summary of the findings is shown in the above figure (Fig 35). The S1P-S1PR1 signalling is abnormally activated in an infection-induced inflammatory disease, i.e. apical periodontitis and this activation partially resulted from the interaction between macrophages and BMSCs in response to infection. The S1P-S1PR1 signalling plays a crucial role in the inflammatory bone remodelling, where on one hand, it stimulates the RANKL production of BMSCs, therefore facilitating osteoclastogenesis, and on the other hand, it induces the differentiation of osteoblasts and inducing osteogenesis. The imbalanced bone remodelling in inflammatory conditions could be induced from the interplay between immune cells and bone cells (osteoclasts and osteoblasts) through the S1P-S1PR1 signalling in regulating both osteoclastogenesis and osteogenesis. This may, therefore, be responsible for both the bone loss and aberrant bone formation seen in inflammatory diseases. These findings

contribute to our knowledge of osteoimmunology, and may also provide new insights that could lead to novel treatment options against bone-destructive diseases.

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Appendices (ethics)

武汉大学口腔医院 医学伦理委员会

科研项目申报审批件 [2013]伦审字（1）号

项目名称	白喉系统调控中性粒细胞胞外陷阱（NETs）与根尖周骨质吸收		
项目负责人	彭桦	项目类别	自主项目
项目承担单位	武汉大学口腔医院		

摘要：本系列研究对进一步阐明慢性根尖周炎发病机制和全面认识中性粒细胞在导致骨质吸收过程中的作用都具有重要意义。

委员签字

姓名	性别	所在单位	伦理委员会职务	意见	签字
樊明文	男	武汉大学口腔医院	主任委员	同意	
杜民科	男	武汉大学口腔医院	副主任委员	同意	
邓旭亮	男	武汉大学口腔医院	委员	同意	
杨华文	男	武汉大学口腔医院	委员	同意	
傅柏平	男	武汉大学口腔医院	委员	同意	
王丽	女	武汉大学口腔医院	委员	同意	
李慧东	女	武汉警官职业学院	委员	同意	
秦绍军	男	北京金台律师事务所	委员	同意	
李柏松	男	湖北正义行律师事务所	委员	同意	

审批意见：

经伦理委员会讨论通过，同意开展研究。

主任委员 樊明文

武汉大学口腔医院医学伦理委员会（印章）

2013 年 3 月 1 日

武汉大学口腔医院医学伦理委员会

科研项目申报审批件

[2011]伦审字（033）号

项目名称：Wnt/β-catenin 信号调控耐受型 DC 与根尖周骨破坏

项目类别：国家自然科学基金面上项目

研究目的：探讨根尖周病的发病机制

研究期限：2012 年 1 月—2015 年 12 月

项目负责人：彭彬

项目承担单位：武汉大学口腔医院

伦理委员会成员	同意	不同意	弃权	签名
樊明文	✓			
杜民权	✓			
雷成家	✓			
杨学文	✓			
傅宏宇	✓			
雷丽	✓			
李慧东	✓			

审批意见：

经伦理委员会讨论通过，同意开展研究。

主任委员签名：

武汉大学口腔医院医学伦理委员会（盖章）

2011 年 3 月 7 日